Towards an endophenotype in multiple sclerosis

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Acknowledgements

"Piglet sidled up to Pooh from behind. "Pooh?" he whispered.
"Yes, Piglet?"
"Nothing," said Piglet, taking Pooh's hand. "I just wanted to be sure of you."

It would not have been possible to produce this thesis without the help and support of so many people, only a few of whom are mentioned below.

Without my primary supervisor, Professor Gavin Giovannoni, this thesis would not have existed. His support, guidance, and seemingly unwavering belief in me throughout my career have been limitless. My second supervisor, Dr Klaus Schmierer has also provided much helpful advice and support, along with Dr Sreeram Ramagopalan. I would also like to thank Jo Topping, whose assistance with the biobanking of samples was invaluable.

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Without the people with MS, their siblings and the healthy controls who took part in this research there would have been nothing. To everyone who provided samples and their time at no benefit to themselves I want to offer a massive thank you.

On a more personal note, I would not have been able to get this far without the support, patience and understanding of those individuals who are closest to me, not only during the good times but also through the not so good. The unconditional love and friendship that I have been shown has made this all possible. Thank you from the bottom of my heart – you know who you are.

Abstract

An endophenotype is a concept that allows the description of complex diseases with genetic and environmental contributions, enabling the identification of an "at risk" population. I aim to describe an endophenotypic gradient between healthy controls, siblings of people with MS and people with MS. Siblings of people with MS are at increased risk of developing MS; this is thought to be a result of genetic and environmental contributions. Epidemiological studies have identified a number of factors contributing to MS risk including smoking, vitamin D, infection with Epstein-Barr virus and HLA-DRB1*1501. A genome wide association study in 2011 gave information regarding the contribution of HLA-type and non-HLA SNPs to MS risk.

I set out to integrate these into an endophenotypic risk score for MS. When the genetic contribution from HLA-DRB1*1501 alone was used, the mean MS risk score was significantly higher for people with MS than siblings or controls. Siblings had a higher MS risk score than controls. The differences between the three groups become more apparent when all genetic information was used in the MS risk score. I used MRI and biomarker studies to validate the MS risk score generated. Preliminary studies enabled an evaluation of the potential association between selected biomarkers and CSF oligoclonal bands.

The analyses performed demonstrate the potential clinical utility of such a score in describing MS risk. Siblings have a risk score intermediate to people with MS and controls, confirming their "at risk" position in the endophenotype construct. Much of the MS risk in siblings can be attributed to genetics, with environmental factors potentially providing the trigger for clinically apparent disease. The findings of this research have the potential to enrich future prevention studies with individuals at high risk of developing MS, enabling such studies to be performed within a realistic timeframe.

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Chapter 1: Introduction

Multiple sclerosis (MS) is the most common non-traumatic cause of neurological disability affecting young people in the developed world (1), with a UK prevalence of approximately 0.4% (MS Society). No single factor appears to precipitate the development of MS; instead a complex interplay of risk factors contributes towards overall risk (2). Epidemiological data implicates both genetic and environmental factors in influencing disease development. There is no cure for MS, and treatment is centred on the modification of disease course. At present, there is no single diagnostic test for MS. The diagnosis is based on clinical findings, supported by paraclinical tests. Non-imaging biomarkers are of limited use in MS, with outcomes in clinical trials predominantly based around clinical or imaging findings.

The dogma that MS results from environmental influences in a genetically susceptible individual is a popular one. To date, there is no evidence to conclusively contradict this hypothesis, and a number of potential environmental contributors to disease development have been identified. If this hypothesis is correct, then in theory at least, it should be possible to prevent MS in a proportion of people who would otherwise go on to develop the disease, through acting to reduce or prevent the impact of these environmental influences. However, designing a study to test such a hypothesis depends on the accurate identification of the “at risk” population.

The endophenotype is a concept that is being increasingly used to describe complex diseases with genetic and environmental influences. It provides a framework through which the population at risk of a complex disease can be described; potentially enabling preventative studies. Identifying and studying individuals at high risk of developing MS provides a powerful opportunity to understand the MS causal cascade and is highly relevant to the development of strategies to prevent this chronic disorder. The endophenotype is a concept that has been used widely in psychiatry to deconstruct complex diseases such as

schizophrenia, by promoting the link between genetic and environmental factors and early disease traits. The endophenotype itself can be thought of as a disease trait, that may take a variety of forms, including neurophysiological, biochemical, cognitive or neuroanatomical. One key feature is that it co-localises with the disease in question. An additional feature of an endophenotype is that it is more common in the siblings of people with the disease in question, highlighting the genetic underpinning of a complex trait.

Siblings of people with MS have an increased risk of developing MS (3). However, it is likely that within this diverse group predictive power can be increased by taking into account genetic and environmental influences on MS risk. Through the construction of a multivariate risk calculator for MS, I hope to be able to identify endophenotypic markers in those siblings who carry a high "risk load" for MS. In theory, those siblings with a high number of MS risk factors should have an increased probability of demonstrating changes in keeping with MS, either on magnetic resonance imaging (MRI) or in terms of biomarker expression.

By studying the unaffected siblings of people with MS within this framework, I hope to be able to demonstrate a gradient of MS risk. The presence of cerebrospinal fluid (CSF) oligoclonal bands in unaffected siblings would provide a powerful endophenotypic maker of disease risk; however CSF analysis of unaffected siblings could not be performed due to ethical constraints. As part of this work it was therefore important to examine the validity of potential biomarkers in a group of patients undergoing lumbar puncture, in order to ascertain whether there was a suitable surrogate marker for CSF oligoclonal bands.

The validity of the endophenotype construct in MS can be assessed using MRI. Unaffected siblings can be assessed for radiological abnormalities in keeping with demyelination; i.e. assessed for the presence of the so-called radiologically isolated syndrome. If a greater proportion of siblings with high loading of MS risk factors show such MRI changes compared

to those thought to be at low risk, this provides powerful evidence in support of the endophenotypic gradient in MS.

This thesis therefore sets out to examine the validity of a number of biomarkers as surrogate markers for CSF oligoclonal bands, and then attempts to both define and validate an risk score for MS, which will in turn be examined using the endophenotype construct. Markers such as lesions seen on T2 weighted MRI and peripheral markers of immune activation will be studied as indicators of the endophenotype. If positive, this study provides both an insight into the chain of events leading to the development of clinical MS, and highlights a number of important avenues for future work.

Chapter 2: CSF oligoclonal bands in MS

2.1. Background

A recent systematic review of diagnostic tests in MS concluded that their efficacy in relation to clinically relevant endpoints remains unclear (4). There is a need for biomarkers that can be used in both the diagnosis and longitudinal monitoring of MS. Although biomarkers do not necessarily have a causal relationship with the disease in question, they often reflect the underlying disease pathogenesis to some degree. In complex diseases such as MS, a biomarker may represent only a single facet of disease pathogenesis (5).

CSF biomarkers have been extensively investigated in multiple sclerosis. CSF IgG oligoclonal bands (OCBs) (figure 2.1; courtesy of Prof. G Giovannoni) represent IgG unique to the cerebrospinal fluid (CSF), i.e. without corresponding IgG in the serum. They are commonly used as part of the diagnostic work-up for MS, but they are not required to make the diagnosis (6). Many studies have been performed examining their frequency, sensitivity and specificity; others have attempted to determine their utility as a prognostic marker.

OCBs are not only found in MS; they can also be found in other inflammatory and infectious diseases affecting the central nervous system, although these can be differentiated from MS using additional CSF and/or clinical findings. OCBs provide evidence of intrathecal IgG synthesis, which is thought to reflect the compartmentalised central nervous system humoral immune activation present in MS. A number of techniques have been developed to detect CSF OCBs. The gold standard is isoelectric focussing (IEF) on agarose gel followed by immunoblotting for IgG (7) using paired CSF and serum. The sensitivity for detection of OCBs is at least 95% using this technique (8-10). Alternative techniques, including silver staining, have reduced sensitivity and specificity in MS (7).

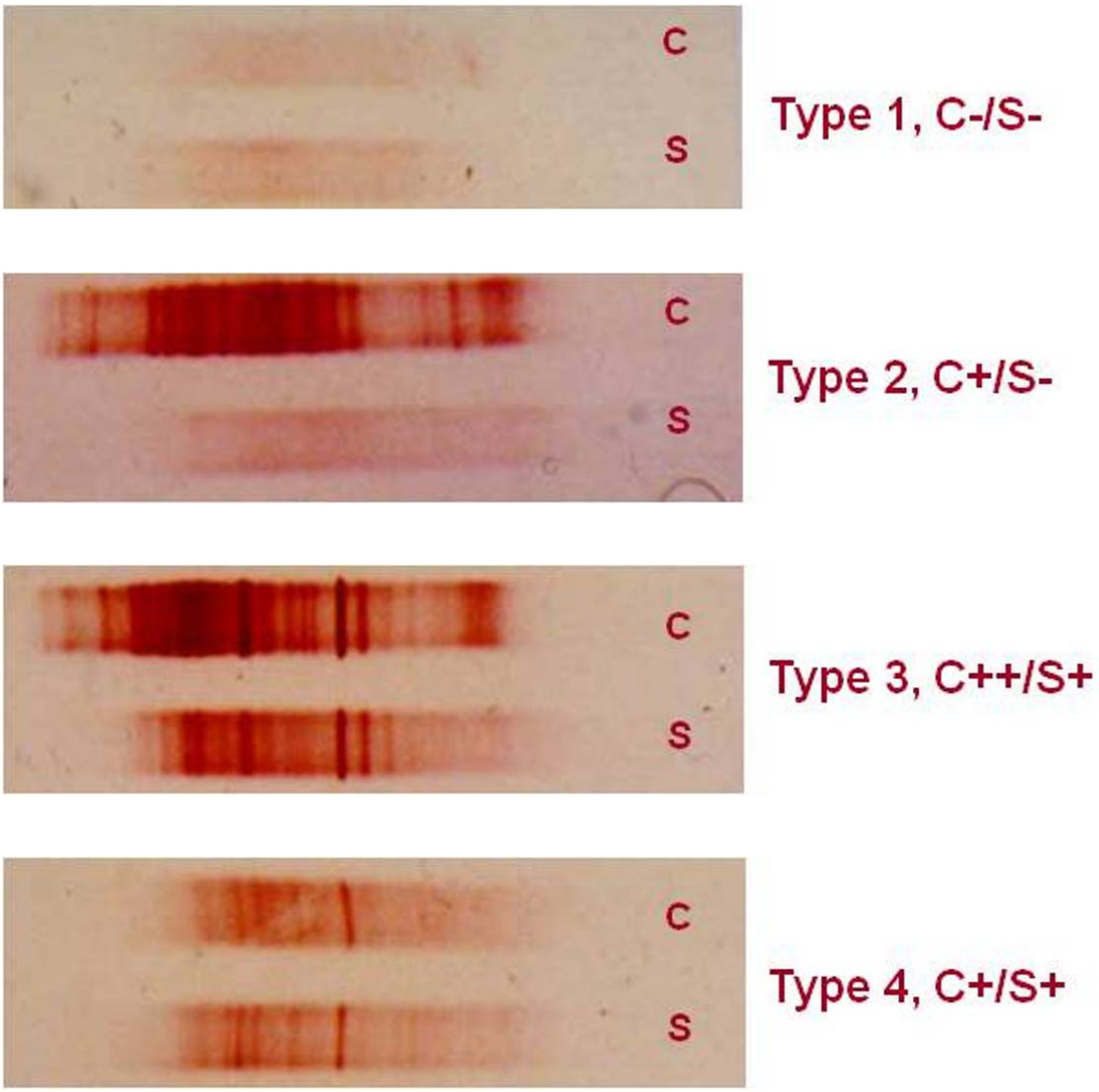


Figure 2.1: Oligoclonal bands unique to the CSF detected by CSF isoelectric focusing with immunoblotting; the oligoclonal bands present are IgG.

There are 4 common patterns: type 1: no bands in cerebrospinal fluid (CSF; C) and serum (S); type 2: oligoclonal IgG bands present in CSF but not in serum, indicative of intrathecal IgG synthesis; type 3: oligoclonal bands in CSF (like type 2) with additional paired identical bands in CSF and the serum sample; the unique CSF bands are indicative of intrathecal IgG synthesis; type 4: identical oligoclonal bands in CSF and the S sample illustrative of a systemic rather than intrathecal immune reaction, with a leaky or normal or abnormal blood–CSF barrier – IgG bands are passively transferred to the CSF.

Whilst CSF oligoclonal bands are frequently used in the diagnostic work-up for MS, they are rarely, if ever, used for disease and/or treatment monitoring; not least because a clinical

response has not been correlated with a quantitative or qualitative change in the number or pattern of oligoclonal bands (11). Additionally, repeated CSF analysis remains an invasive method of monitoring. Despite improvements in lumbar puncture method and technique there is significant resistance from patients to repeated lumbar puncture. The absence of a sensitive and specific serum antibody in MS (12) has limited the use of serological biomarkers, although the search continues.

2.1.1. CSF oligoclonal bands

The precise prevalence of OCB positivity in MS is not known. There are a large number of studies examining this as either a primary or secondary research question. The largest of these studies enrolled around 3000 patients (13). Whilst the advent of MS databases has enabled large population-based studies, there are limitations in using these to examine OCB prevalence in MS, as different centres may use different techniques for detecting OCBs.

There are also a number of studies examining the prognostic significance of OCB positivity (and negativity) when assessing a patient with a clinically isolated syndrome (CIS). The presence or absence of OCBs can give important information regarding the likelihood of progression to clinically definite MS (14), however, the magnitude of this prognostication has yet to be determined in a population size greater than 500.

The relationship between the presence or absence of OCBs and disease outcomes in those with clinically definite MS is an area of interest. To date, there is no definitive answer as to whether the presence of OCBs confers a better outcome in terms of disability progression. Studies have both confirmed and refuted this theory.

It has recently been suggested that the latitude at which a patient resides is related to the probability of that patient being OCB positive (13). The reasons behind this variability are unclear, and this finding requires replication.

Given the volume of literature that has previously been published surrounding OCBs and MS and the variation in techniques that have been used, there is a need to clarify the use of OCBs in the clinical arena, and the first part of this study sets out to do this.

2.1.2. Aims

Given the lack of precise information surrounding the prevalence of OCB in MS, a primary aim was to calculate the most accurate estimate of OCB prevalence in both MS and CIS produced to date. Through a systematic review of the literature and a meta-analysis of the results contained within this domain, the prevalence of OCBs in both clinically definite MS and CIS are clarified. The relationship between OCB positivity and MS disease type, conversion from CIS to clinically definite MS, and any potential relationship between OCB status and latitude in MS and CIS are also examined.

2.2. Calculating the precise prevalence of CSF oligoclonal bands in MS

2.2.1. Methods

2.2.1.1. Search strategy

PubMed was searched using the terms “multiple sclerosis and oligoclonal bands”, “multiple sclerosis and OCBs”, “multiple sclerosis and OCB”, “clinically isolated syndrome and oligoclonal bands”, “clinically isolated syndrome and OCB” and “clinically isolated syndrome and OCBs” on 22nd October 2012. The resulting abstracts were hand-searched for publications meeting the inclusion criteria (see section 2.2.1.2.). The results from each search were cross-referenced as many duplicate results were identified.

2.2.1.2. Inclusion criteria

Inclusion criteria were pre-specified. The search was limited to papers published after 1980, as IEF with immunofixation was not in widespread use before this time. In order to be included, papers had to include CSF data on a minimum of 10 patients with either MS, suspected MS, or CIS. Those papers that specified the inclusion of patients with neuromyelitis optica (NMO; Devic's disease) were excluded. Studies including patients with "Asian optico-spinal MS" were not excluded from the initial analysis, as this diagnostic entity appears to overlap with MS. A conservative analysis later excluded those studies including patients with Asian optic-spinal MS. In studies that included both patients with MS and CIS, the two groups had to be clearly separated.

The number of patients found to be OCB positive and negative had to be clearly stated. The technique used to determine OCBs was recorded. For those studies initially used to determine the prevalence of OCB in MS, the study had to specify that OCBs were determined by IEF with immunofixation; studies using alternative techniques were excluded from this main analysis. A supplementary analysis where all papers were included, regardless of method of OCB detection were included was also performed. Those studies that used a small number (<20) of patients with the primary aim of comparing methods for detecting OCBs were rejected at this stage.

In order to be included in the prognosis data analysis, papers had to give data regarding both the number of OCB positive and negative patients who met a pre-defined clinical end point. Data regarding all end points were gathered. Data from studies using all techniques for OCB detection and from those not specifying methods were recorded. Those studies giving narrative information about clinical outcomes were also recorded, but they could not be included in the formal data analysis.

2.2.1.3. Statistical analysis

Prevalence, sensitivity and specificity, and positive and negative predictive values were calculated using standard formulae. The odds ratio (OR) of clinical outcomes according to OCB status was calculated using the generic inverse variance model in RevMan 5.1 (Cochrane collaboration). A random effects model was applied unless I^2 was ≤25%; in which case a fixed effects model was used (15). Between-study heterogeneity was assessed using Cochran's Q chi-square test and I^2 (16). Bias was assessed using visual inspection of funnel plots and quantified using an Egger p-value (17).

When examining clinical outcomes, those studies using IEF with immunofixation were initially studied in isolation. Studies using other techniques were then added to the cohort in order to increase the number of patients analysed. Separate analyses were performed for MS and CIS. When examining outcomes in CIS two analyses were performed – one with all patients with CIS, and a subgroup analysis of patients presenting with optic neuritis (ON). Fisher's exact test was used to compare the proportion of OCB positive and negative patients reaching the clinical outcomes. Linear regression (modelled using PASW v18 (SPSS)) was used to examine whether any relationship existed between conversion rate and duration of follow up in both the OCB positive and OCB negative groups.

2.2.1.4. Effect of latitude on oligoclonal band status

The location of each study was determined using the data provided in the manuscript, and the latitude determined using Google maps (www.maps.google.com). Where samples had been taken from a regional or national cohort, the latitude of the midpoint of that country or area was used for the analysis. Papers describing samples taken from international collaborations were excluded from this analysis. MS and CIS were analysed separately.

A linear regression model was used for this analysis (PASW v18 (SPSS)). The proportion of CSF samples found to be OCB positive were regressed on the population latitude. The dependent variable was the proportion of OCB positive samples, and the independent variable latitude, and the contribution of latitude to the equation *O:E ≈(latitude*X)+constant* was assessed. An additional independent variable for sample size was then added to the model in order to assess whether this affected the results obtained.

2.2.2. Results

2.2.2.1. Included papers

Following the initial screening 350 unique papers were identified. The abstracts and full text of these papers were then hand searched for papers meeting the inclusion criteria. 71 articles were selected for inclusion in the final analysis (see supplementary appendix 2). The reasons for rejecting papers at this stage were varied, but most commonly included papers that selected small numbers of patients for methodological studies (n=58), papers that selected OCB positive or negative patients only (n=15), review papers (n=18) and papers examining IgM OCB only (n=12). The selection process is summarised in figure 2.2.

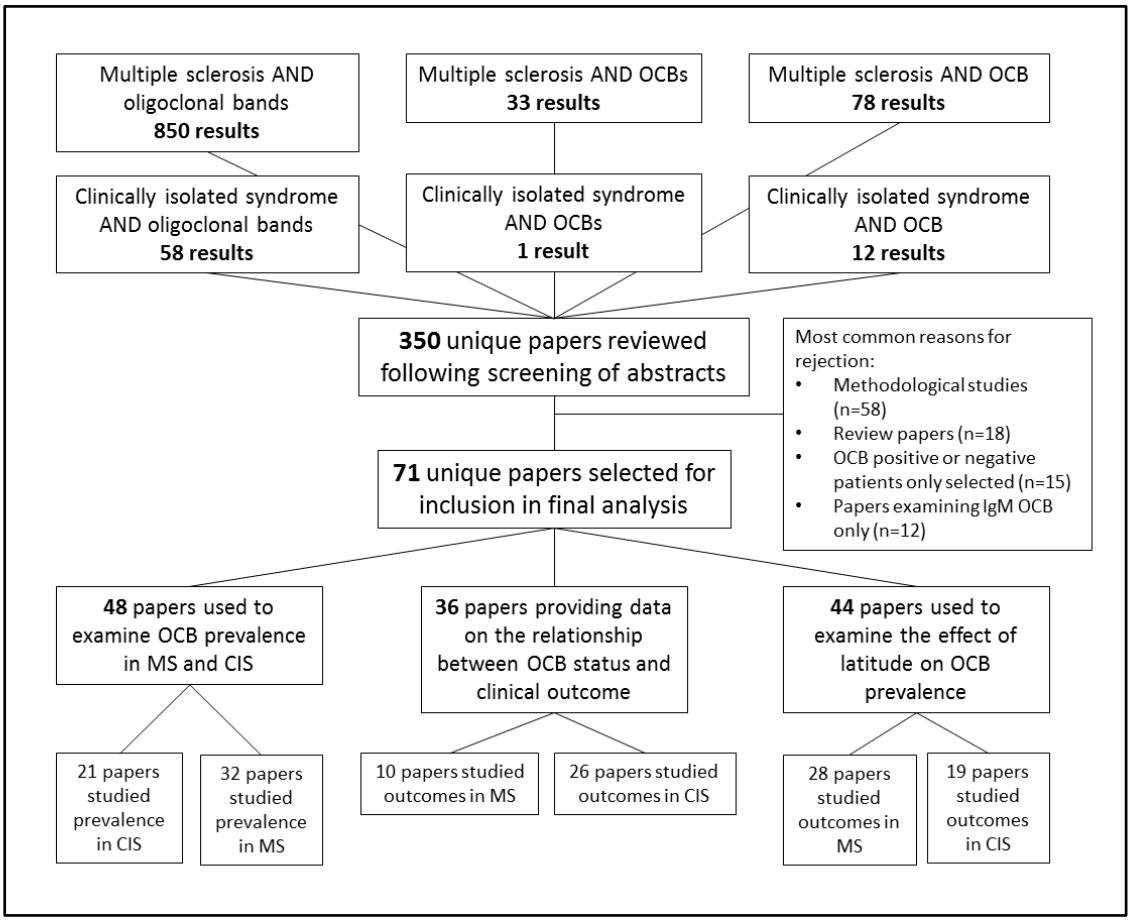


Figure 2.2: Study selection

48 studies were used to assess the prevalence of OCB in MS and CIS, 36 were used to calculate the association between OCB and clinical outcomes, and 14 papers gave qualitative information regarding the relationship between clinical outcomes and OCB status (see supplementary appendix 2). Of the 36 papers used to calculate outcomes, 18 used IEF with immunofixation, in 12 the technique was not specified, and the remaining 6 used a specified technique other than IEF with immunofixation, most commonly electrophoresis with silver staining. 10 studies used in the clinical outcomes data analysis studied outcomes in MS; and of the remaining 26 examining CIS, 9 selected patients with ON. 44 papers were used in the latitudinal analysis; 28 to determine the relationship between latitude and OCB status in MS and 19 in CIS.

2.2.2.2. Oligoclonal band prevalence in MS and CIS

There were OCB data meeting the inclusion criteria in a total of 12,253 MS patients, 10,751 of whom were OCB positive and 1577 OCB negative; overall 87.7% patients with MS were OCB positive. When the three Asian studies (18-20) were excluded, 10,719/12,171 (88.1%) MS patients were found to be OCB positive. When all studies were included, regardless of population and technique used to detect OCB, 16,678/19,773 MS patients were OCB positive (84.3%). A conservative analysis, where only those papers using IEF with immunofixation were included, and all papers possibly using duplicate cases (i.e. those originating from the same centre) and the Asian studies were excluded showed 5495/6118 (89.8%) patients with MS were OCB positive.

There were OCB data meeting the inclusion criteria in a total of 2685 patients with CIS, of whom 1841 were OCB positive and 844 OCB negative; overall 68.6% patients with CIS were OCB positive. There were no studies examining OCBs in CIS in Asian patients. When all studies were included regardless of technique, 3580/5154 (69.5%) patients were OCB positive. A conservative analysis, with exclusion of all possible duplicate datasets, showed 1489/2205 (67.5%) CIS patients to be OCB positive.

2.2.2.3. Relationship between oligoclonal band status and clinical outcomes in MS

10 studies gave data regarding clinical outcomes in patients with MS. Of these, 4 used IEF with immunofixation (21-24). In all of the studies using IEF with immunofixation, expanded disability status score (EDSS) related outcome measures were used to define clinical outcomes; one used EDSS of 4 at 10 years disease duration (21), two used EDSS 6 during follow up (22, 23), and one an increase of ≥1 EDSS point in 5 years (24). When the results were combined, 667/1764 (37.8%) OCB positive patients reached the specified disability outcome compared to 42/154 (27.2%) OCB negative patients ($p<0.0001$, Fisher's exact test). When the meta-analysis was performed this gave an odds ratio (OR) of reaching the disability outcome of 1.96 (95%CI 1.31-2.94; $p=0.001$) with no between study heterogeneity ($I^2=0\%$; $X^2=2.95$, df=3, $p=0.40$) (figure 2.3). There was no significant publication bias (Egger p-value=0.12). A subgroup analysis of the two studies using EDSS 6 as an endpoint (22, 23) gave an OR of reaching EDSS 6 of 2.03 (95%CI 1.24-3.33; $p=0.005$) with no heterogeneity ($I^2=0\%$; $X^2=0.87$, df=1, $p=0.35$).

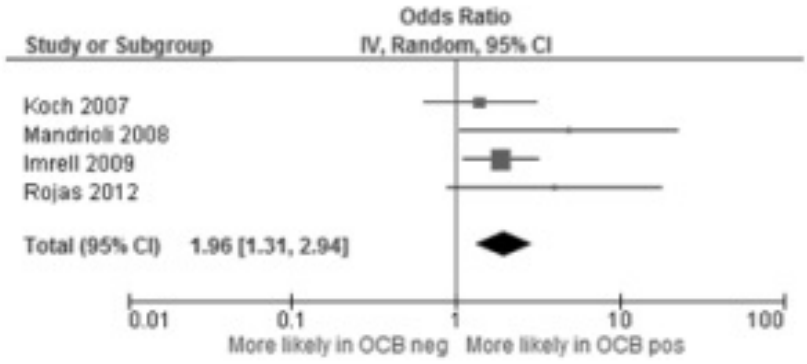


Figure 2.3: Relationship between OCB status and clinical outcomes in MS

When the six studies using other techniques for measuring OCBs were included (see supplementary appendix 2), the range of outcome measures used increased. EDSS was used to define the endpoint in a number of studies; with outcomes including EDSS 6, 7.5, or 8 at between 5 and 10 years disease duration. One study used worsening of EDSS by 1 point over 2 years, and one study used "poor recovery from relapses". 770/2202 (35.0%) OCB positive patients reached the defined disability outcome measure compared to 66/333 (19.8%) OCB negative patients ($p<0.0001$, Fisher's exact test). Inclusion of those studies using alternative techniques gave an OR of meeting the study endpoint of 1.65 (95%CI 1.27-2.13; $p=0.0002$) with moderate heterogeneity ($I^2=48\%$; $X^2=0.22.97$, $df=13$, $p=0.03$) (data not shown). This was not significantly different from the result obtained when only those studies using IEF with immunofixation were included.

13 studies gave narrative results without absolute numbers. One study found a significantly lower relapse rate in OCB negative patients (relapse rate 1.45±0.69 in OCB positive and 0.58±0.64 in OCB negative, $p=0.001$) (25). None of the other studies demonstrated any relationship between the presence of OCBs and the disability outcomes collected, including relapse rate (26-28), EDSS (14, 25-30), and MS severity score (26, 31, 32).

2.2.2.4. Relationship between OCB status and outcomes in CIS

14 studies examined the relationship between OCB detected by IEF with immunofixation and outcomes in CIS. 2 of these studies specified ON (33, 34), and one a brainstem syndrome as the CIS (35). 12 studies used conversion to CDMS as the outcome, 1 used radiological conversion to MS (33) and one used the number of patients reaching EDSS 6 at 5 years (13). The study using EDSS 6 as the outcome measures was excluded given the very different outcome measure, leaving 13 studies in the analysis (see supplementary appendix 2). 733/1143 (64.1%) OCB positive patients converted to MS compared to 139/616 (22.6%) OCB negative patients ($p<0.0001$, Fisher's exact test). This gave a sensitivity of 0.84 and a specificity of 0.54 when using OCB to predict conversion to CDMS. The positive predictive value (PPV) was 0.64 and the negative predictive value (NPV) 0.77. Studies followed patients up for a variable amount of time, ranging from 18 months to 8 years.

When the meta-analysis was performed there was an OR of conversion to MS of 9.88 (95%CI 5.44-17.94; $p<0.00001$) in the OCB positive patients (figure 2.4). However there was significant between study heterogeneity ($I^2=71\%$; $X^2=40.79$, df=12, $p<0.0001$). There was no evidence of publication bias (Egger p-value=0.20). Excluding the study using radiological conversion (33) did not significantly alter these results. Other attempts to explore the underlying causes of the heterogeneity observed were similarly unsuccessful.

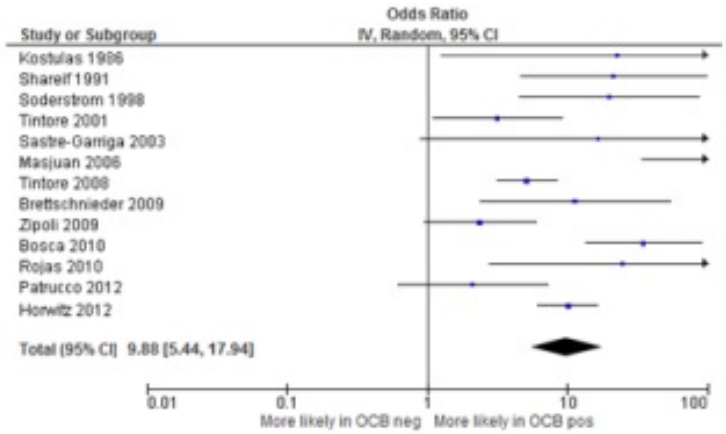


Figure 2.4: Relationship between OCB status and conversion to MS in CIS

When all of the studies examining the relationship between OCB and conversion to MS (regardless of the technique used to detect OCB) were considered (an additional 12 studies; see supplementary appendix 2), 973/1584 (61.4%) OCB positive CIS patients converted to MS compared to 173/927 (18.7%) OCB negative CIS patients ($p<0.0001$, Fisher's exact test). This gave an OR of conversion to MS of 9.99 (95%CI 6.54-15.27; $p<0.00001$) in the OCB positive patients (data not shown). There was significant between study heterogeneity ($I^2=57\%$; $X^2=56.27$, df=24, $p=0.0002$) which proved impossible to eliminate. This result was not significantly different from that obtained when only those studies using IEF with immunofixation were used.

Given the large number of studies examining outcomes in optic neuritis, a sub-group analysis of these studies was performed. The majority of studies (7/9) did not specify that IEF with immunofixation had been used. The results were similar to those obtained when all CIS were considered, with 474/743 (63.8%) OCB positive patients developing MS compared to 98/429 (22.8%) OCB negative patients ($p<0.0001$, Fisher's exact test). The meta-analysis gave an OR of conversion to MS of 10.13 (95%CI 7.11-14.44; $p<0.00001$) in those patients who were OCB positive, with no heterogeneity ($I^2=0\%$; $X^2=6.96$, df=8, $p=0.54$).

When all studies were included there appeared to be a relationship between the proportion of OCB positive patients converting to CDMS and the duration of follow up (using linear regression, p=0.042, R^2=0.1833) (figure 2.5). However, when only those studies using IEF with immunofixation were included this relationship was no longer significant. It therefore remains unclear if all OCB positive patients would convert to CDMS if followed up for an infinite amount of time. Additionally, MRI data was not examined as a covariable in this study. There was no relationship between the proportion of OCB negative patients converting to CDMS and the duration of follow up. Given the low conversion rate in the OCB negative group together with the lack of any relationship between conversion rate and duration of follow up in the OCB negative group, it was not possible to determine whether conversion occurs sooner in those who are OCB positive.

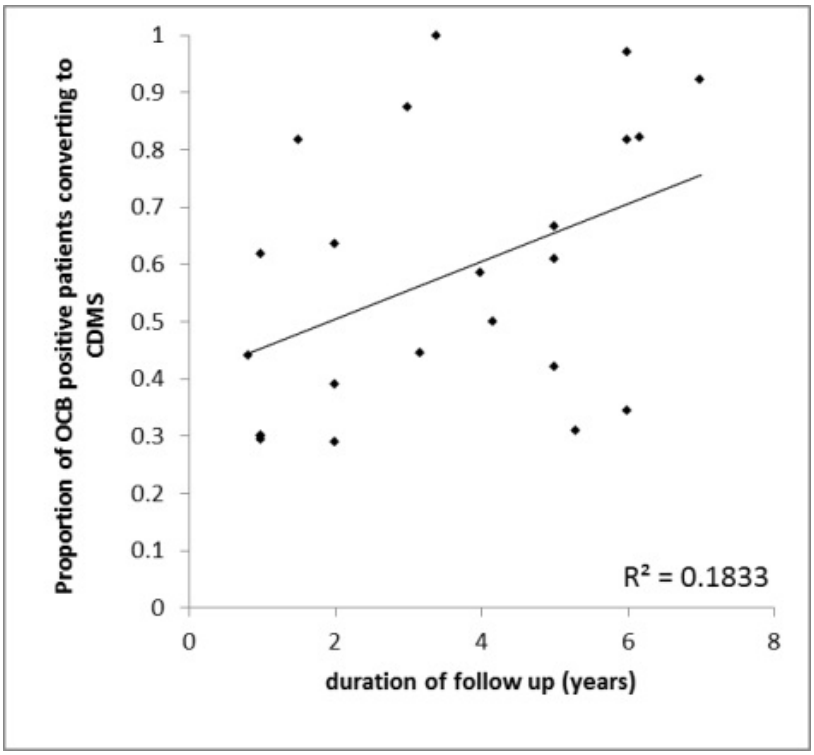


Figure 2.5: Graph demonstrating the relationship between duration of follow up and conversion rates in those who are oligoclonal band positive (data from all studies).

2.2.2.5. Relationship between oligoclonal bands and latitude

28 studies with data on OCB in MS were used to determine the effect of latitude on the proportion of MS samples positive for OCBs (see supplementary appendix 2). Only those studies using IEF with immunofixation were included in this section of the analysis. Linear regression revealed a significant relationship between OCB positivity and latitude (p=0.002, figure 2.6) with a correlation coefficient (R^2) of 0.31. This relationship was maintained when an additional variable for sample size was included in the model (p for effect of latitude=0.009, p for effect of sample size=0.833). The single outlying point with a very low rate of OCB positivity originated from Taipei, Taiwan. When Asian studies were excluded, a significant relationship remained (p=0.005 in the linear regression model; R^2=0.169).

19 studies were included in the latitudinal regression model for CIS. There was no significant relationship between the proportion of OCB positive samples and latitude (p=0.099, data not shown); this was not altered by the inclusion of sample size in the model (p for effect of latitude=0.119, p for effect of sample size=0.856).

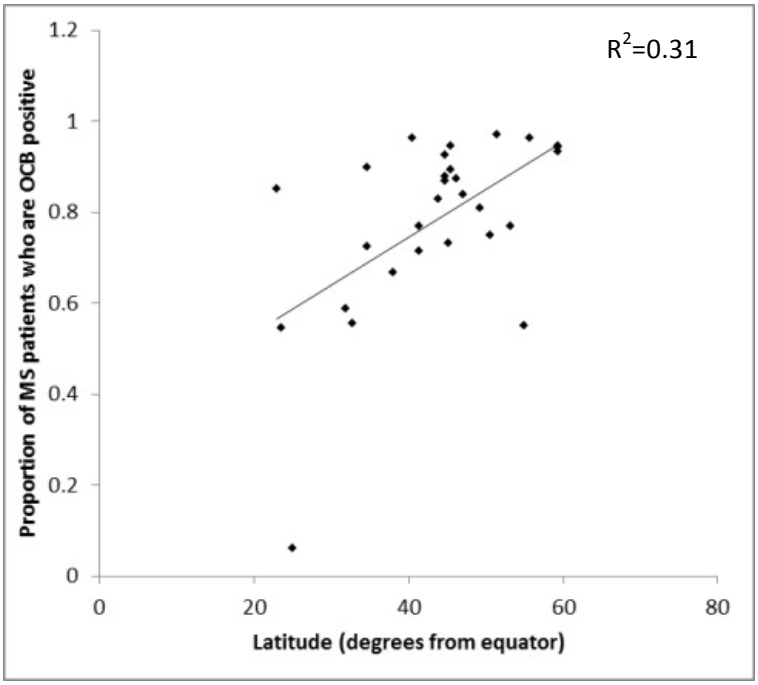


Figure 2.6: Graph demonstrating the relationship between latitude and the proportion of MS patients who are OCB positive

2.2.3. Conclusions

By pooling a large number of studies with information regarding OCB status in MS and CIS, this study is able to inform clinicians regarding the clinical significance of OCBs in patients with both suspected and definite MS. Just under 90% patients with MS and around 68% CIS patients are OCB positive. In my opinion, this supports the hypothesis that "OCB negative MS" is a disease entity separate to the majority of people with MS, and indeed may well not represent MS as we pathologically understand it at the present time. The increasing recognition of neuromyelitis optica (NMO) as a separate disease to MS, with different pathology and response to treatment, would support this hypothesis – until relatively recently patients who are now diagnosed with NMO would have been diagnosed with "OCB negative optico-spinal MS", a label that is no longer used. In patients with CIS, the presence of OCBs is associated with a markedly increased risk of conversion to MS. The magnitude of this risk equates to an OR of 9.9, seemingly regardless of the anatomical location of the CIS.

The proportion of the ~10% patients who have been diagnosed with "OCB negative MS" that actually have MS remains unclear. It is likely that at least some of these patients will not actually have pathologically definite MS; 95% surveyed US neurologists felt that they had evaluated a misdiagnosed patient in the past year (36). The most common suspected alternative diagnoses included nonspecific white matter abnormalities on MRI, small vessel ischaemic disease and migraine (36). All of these conditions can cause MRI abnormalities that may be mistaken for MS; however none are associated with CSF OCB. Solomon et al (36) argue that the inappropriate use of imaging criteria for diagnosis may play an important role in misdiagnosis. Given the high rate of OCB positivity in this meta-analysis, it is clear that OCB positivity plays an important role in MS diagnosis, and those patients who are OCB negative should have their diagnosis closely considered.

The relationship between the presence of OCB and clinical outcomes in those people who have developed MS is less clear, with the heterogeneity between outcome measures used, length of follow-up and publication/reporting bias clouding the analysis. Whilst the initial analysis would suggest that those patients with MS who are OCB positive have an OR of 1.96 of reaching specified disability outcomes at follow-up compared to those who are OCB negative, this must be qualified by examining the studies giving narrative negative results. Whilst the negative studies in general did not provide raw data regarding the number of patients reaching disability milestones, they must be taken into account.

The confirmation of the finding that latitude does appear to be associated with OCB status in MS (but not in CIS) is interesting. In theory, the relationship between OCB positivity rate and latitude in people with MS should be independent of the prevalence of MS if all people with a clinical diagnosis of MS actually have MS. However, if one assumes that the incorrect diagnosis of MS, leading to a diagnosis of "OCB negative MS" is one of the major causes of OCB negative MS; and this is in turn dependent on the prevalence of MS, then this would have a greater effect on the perceived OCB prevalence in those countries with lower rates of MS. Thus a potential factor underlying the relationship between OCB positivity and latitude would be a higher rate of MS misdiagnosis relative to absolute MS prevalence in those countries where MS is rarer.

MS severity is unlikely to underly the trend in OCB positivity rates varying with latitude. There is no convincing evidence that OCB positive MS confers a worse prognosis than OCB negative MS (if such an entity exists) – whilst the meta analysis suggested that this might be the case, the discrepancy between clear reporting of positive results and non-reporting negative results (which were alluded to in the discussion sections of various papers) lead me to believe that there is no clear prognostic significance to OCB once a diagnosis of clinically definite MS has been reached.

It has previously been shown that in the Northern Hemisphere the prevalence, but not the incidence, of MS varies in a latitudinal manner (37). The reasons for this difference are unclear, but may reflect changing cultural habits, including attitudes to sunbathing and sunscreen, which in turn affect population vitamin D levels (37). The latitudinal variation in OCBs may therefore reflect a shared underlying aetiology with the variation in prevalence rates, and it will be interesting to see if this gradient changes or disappears altogether.

Further studies are therefore required in order to determine why latitude appears to affect OCB status in this way. The relationship between OCB status and clinical outcomes in MS remains unclear, and large scale prospective studies are required in order to overcome the significant selection and publication bias that may underlie many of the results in the literature to date.

Chapter 3: Urinary biomarkers in MS

3.1. Introduction

Urine provides a valuable fluid readily available for serial sampling in MS, but it is understudied and underutilised (38). Urine has the distinct advantage of being easy to collect, with frequent and repeated sampling easy to achieve. In addition, substances excreted in the urine are often present in higher concentrations than in CSF or blood due to fractional excretion; a result of both glomerular filtration and water resorbtion in the renal tubule (39). A single urine sample represents urinary excretion over a number of hours due to urine storage in the bladder (39).

Whilst some work has been done on urinary biomarkers in multiple sclerosis, these have not been extensively studied and validated. The reasons behind this are unclear; concerns regarding frequent urinary tract infections in people with MS limiting the utility of urinary markers may be one explanation. However, with the ready availability of bedside tests for urinary tract infection (such as the presence of nitrites), samples can be easily evaluated for evidence of asymptomatic infection prior to further analysis. To date there are no longitudinal studies evaluating the use of urinary biomarkers as surrogate markers against other disease outcome measures in MS. There remains a need for sensitive and reliable surrogate markers for the monitoring of disease activity in MS, and it may well be that the time has come to revisit urine as a potential source of such biomarkers. The second part of this study was to examine selected potential urinary biomarkers in MS, and define their relationship to CSF biomarkers.

3.1.1. Urinary free light chains

People with MS have increased levels of immunoglobulin free light chains (FLC) in their CSF compared to healthy controls (40). These excess CSF FLC, which are produced by B-cells and plasma cells within the central nervous system, can be detected using ELISA (41). CSF κ FLC have been shown to correlate with disability progression (42). However, FLC cannot be detected in the serum of people with MS (43, 44), either as a result of dilution or rapid fractional excretion by the kidneys.

It has been known for many years that FLC are present at increased concentrations in the urine of people with MS (41, 43, 45), and these can be detected with either a spot urine sample or 24-hour urine collection (43). Urinary FLC are significantly increased in people with clinically isolated syndrome, relapsing remitting and progressive MS, with no significant difference between these groups (41). The elevated levels of urinary FLC seen in MS are not a disease specific phenomenon; levels are also increased in HIV, rheumatoid arthritis and posterior uveitis (41). There does appear to be a change in urinary FLC levels with disease modifying treatment. When urinary FLC were determined in a cohort of patients with primary progressive multiple sclerosis participating in a trial of interferon-β in primary progressive MS, those patients who completed 24 months of treatment with interferon-β1a had lower urinary FLC at 15 and 24 months than those receiving placebo (46). There was a statistically significant relationship between percentage change in spinal cord volume and change in urinary FLC between 12-24 months, however no significant relationships between urinary FLC and any clinical or other radiological outcome measures were determined (46).

To date, the presence of urinary free κ and λ free light chains has not been studied in relation to the presence of CSF OCBs. Urinary FLC show considerable potential as a biomarker in MS. However, much work remains to be done. It is not known whether urinary

FLC change over time, and whether levels are affected by the various treatments available for relapsing remitting MS. It is also not known whether they correlate with other markers of disease activity. However, they are deserving of further study.

3.1.2. Other urinary biomarkers

3.1.2.1. Neopterin

Neopterin is a product of interferon-γ activated macrophages (47), and its formation is augmented by the presence of TNF-α (48). Neopterin has been extensively studied in the context of systemic infections, HIV, malignancies and autoimmunity (49). In MS, increased neopterin levels have been found in the CSF (50-52) and serum (51). Serum neopterin levels have been used to monitor the biological effect of interferon-β (53, 54); levels peak approximately 2 days post-dose (54, 55). No studies have examined a possible correlation between CSF and urinary neopterin levels. A correlation has not been found in other disorders, although in these studies urine concentration was not controlled for, making the results difficult to interpret (56).

Neopterin is a stable compound in vivo, which is excreted in the urine. When measured in urine it should be expressed as a ratio to creatinine (or total protein) in order to control for urine concentration (57). It can be measured using high pressure liquid chromatography (HPLC), a standard technique (57, 58), or ELISA. Neopterin is stable in urine, with reproducible levels measured when urine is stored at room temperature for 48 hours, ≥72 hrs at 4^{o}c and ≥ 4 months at -20^{o}c (57). In addition, neopterin levels remain stable through repeated free-thaw cycles (57). However, neopterin is light sensitive, and so care should be taken to shield samples from light (57).

Urinary neopterin (expressed as neopterin:creatinine ratio) is higher in people with MS than in healthy controls (57, 59), with an mean level of 187 µmol/l (95% CI: 165–277) in people with relapsing remitting MS, 218 µmol/l (164–517) in secondary progressive MS and 187 µmol/l (135–231) in primary progressive MS compared to 134 µmol/l (97–152) in healthy controls reported in one study (57). The sensitivity and specificity of neopterin levels greater

than a given threshold have not been calculated. There is a lack of specificity in raised urinary neopterin – levels rise in the context of a systemic inflammatory response, such as viral infection (57). In addition, neopterin levels show increased day-to-day variability in people with MS, which is thought to reflect fluctuations in inflammatory activity, making single readings difficult to interpret (57).

As individual neopterin levels fluctuate in response to stimuli such as infection and inflammation, there is utility in repeated measurements in the context of MS. In a longitudinal study, 29/31 (94%) MS patients demonstrated increased neopterin excretion compared to healthy controls during the course of the study (57) as opposed to 39/106 (37%) when levels were measured at a single time point in a cross-sectional study (60). A possible trend for people with secondary progressive MS to have both higher mean urine neopterin and greater intra-patient variability than those with relapsing remitting MS has been noted (57), although these findings have not been reproduced. This observation did not reach statistical significance, and there were only a very small number of patients in this study (10 with relapsing remitting MS and 11 with secondary progressive MS).

There may be an increase in urinary neopterin levels prior to clinical relapse in people with MS, and this increase appears to be of a greater magnitude than the “normal” day-to-day variability in MS (57). However, this did not reach statistical significance when compared to the background variability, possibly due to the small number of patients that had a clinical relapse in the single study examining this relationship (57). There was no correlation between the clinical severity of relapse and urinary neopterin levels (57). Urine neopterin levels appear to fall with intravenous steroid treatment (57), however this finding has only been demonstrated in two patients, so it must be interpreted with caution. Although serum neopterin levels rise in response to infection (49), changes in urinary neopterin in response

to infection in people with MS was found to be variable, so no firm conclusions can be drawn regarding this (57).

Neopterin therefore shows promise as a potential urinary biomarker in MS, although further work is needed in order to examine longitudinal changes both with and without treatment. Fluctuations in neopterin levels, and the lack of specificity of increased levels may hinder its use, although it may well be possible to overcome these through repeated sampling. In addition, correlation with existing clinical and MRI outcome measures is required, together with further studies examining how relapses affect urinary neopterin levels. There are certainly aspects of urinary neopterin levels in the context of MS that justify its inclusion in further avenues of study.

3.1.2.2. Nitric oxide metabolites

Increased levels of nitric oxide (NO) and nitric oxide metabolites have been found in the CSF (61-64) and serum (62, 65) of people with MS. NO is neurotoxic, and it has been hypothesised that this neurotoxicity may play a role in MS disease progression (60). In experimental allergic encephalomyelitis (EAE), an animal model of MS, NO appears to play a role in central nervous system inflammation (66).

Urinary nitrate and nitrite excretion are only crude indices of endogenous NO production, as a large proportion of NO is consumed by nitrosylation reactions in vivo (67). In addition, nitrates are produced by gastrointestinal bacteria and nitrate levels are affected by diet (68, 69). NO metabolites can be easily measured in the urine using a modified nitrate reductase and Griess reaction method (70). By measuring either urinary total protein or creatinine, and expressing levels of NO metabolites as a ratio to protein, urine concentration can be controlled for (57).

Urinary NO metabolites are significantly elevated in people with MS (60) compared to healthy controls, however, they are also elevated in patients with rheumatoid arthritis and HIV (60). There is no significant difference in urinary NO metabolites between different MS disease subtypes (60). Additionally, levels are not associated with either clinical or MRI measurements of disease activity or severity, and there is no relationship between urinary NO metabolites and disability in primary progressive MS (71). Urinary NO metabolites do not change with clinical relapse (60) or treatment with interferon-β (71).

Nitric oxide metabolites do not show the same promise as urinary FLC or neopterin. It has been established that they do not change with clinical relapse or treatment, limiting their utility. In addition, the level of NO metabolites may be significantly altered by urinary tract

infection, and urinary levels of NO metabolites are likely to represent a crude measure of MS disease activity at best.

3.1.2.3. Other potential avenues for study

Ubiquitin C-terminal hydrolase L1 (UCHL1, previously known as PGP9.5) is a proteolytically stable, small protein of neuronal origin with a molecular weight of 24.5kDa (72). UCHL1 levels are increased in both serum and CSF following traumatic brain injury (73), and they correlate with both severity of injury and long term outcomes (73, 74). CSF UCHL1 is elevated for a number of days following aneurysmal sub-arachnoid haemorrhage (72), with dynamics mirroring those of CSF heavy chain neurofilament (NFh). As the molecular weight of UCHL1 is <30kDa, it is likely to be freely filtered at the glomerulus; hence it should be detected in urine when serum levels are elevated. However, there have been no previous attempts to detect or quantify this protein in the urine.

A single paper has examined heavy metal excretion in MS (75). Urinary iron was significantly higher in MS than controls. On subgroup analysis, people with secondary progressive MS had significantly higher levels of urinary aluminium and iron than controls, however patients with relapsing remitting MS did not significantly differ from controls (75). Conversely, median urine silicon concentration was significantly lower in secondary progressive MS than in controls. People with MS showed a wide range of concentrations of these metals in their urine (75). The significance of either higher levels of urinary iron and aluminium excretion or lower levels of silicon excretion in MS is unknown, and these changes have not been correlated with other markers of disease severity (75). These findings have been neither replicated nor refuted.

There are inflammatory markers that have been detected in the serum of people with MS and that are known to be detectable in the urine, that have not yet been studied in people with MS. β_2-microglobulin forms the 12 kDa light chain of the class I major histocompatibility complex (MHC-I) on the surface of many cells, including human peripheral blood cells (76).

Elevated serum levels of β_2-microglobulin are thought to reflect increased lymphocyte turnover (77), and β_2-microglobulin can be detected in the urine using ELISA (78). Some have found elevated CSF (79) levels of β_2-microglobulin in MS, although this result has not been consistently replicated (51, 80). IL-1, IL-2, IL-6 and IL-8 can also be detected in the urine (39). Much remains to be discovered about these molecules in MS, and urine may well provide a useful avenue for study in the future.

3.2. Methods

With existing data demonstrating elevated levels of free immunoglobulin light chains and neopterin in the urine of people with MS, together with the total lack of data examining any link between CSF and urinary levels, the decision was taken to focus on these two potential biomarkers. In addition, given the potential utility of UCHL1, and the paucity of information regarding its presence or absence in either the CSF or urine in MS, an additional study of this compound was made. All of these potential biomarkers are relatively stable compounds, and thus carry good potential for practical utility in diagnosis and/or monitoring in MS.

3.2.1. Patient recruitment

This project had ethical approval from the Outer North London Ethics Committee (Ref 10/H0724/36). Patients (n=39) were recruited from the Neurology day-case unit at the Royal London Hospital. Patients who were undergoing a diagnostic and/or therapeutic lumbar puncture which included an assessment for CSF OCBs as part of their routine care were approached about the study. Patients who had received recent (within 3 months) corticosteroid treatment were excluded from this study. All patients who had OCBs present in the CSF had MRI evidence of multiple lesions with dissemination in space, and were therefore regarded as “early MS” for the purposes of this study.

3.2.2. Sample collection

CSF samples were obtained at the time of lumbar puncture. An extra volume of up to 5ml CSF was taken at the time of lumbar puncture. Mid-stream urine samples were obtained on the same day. All samples were collected in sterile containers with no additives. Urine samples were assessed for the presence of nitrites (in order to exclude bacterial infection) at the time of sampling. Samples were coded and anonymised, divided into aliquots and stored at -80^{o}c on the day of sampling. Care was taken not to expose samples to direct light for prolonged periods of time in order to allow neopterin levels to be accurately assessed. Patient details are given in table 3.1.

Table 3.1: Patient details

	OCB positive	OCB negative
N (female; %)	16 (9; 56%)	23 (16; 70%)
Age (mean; range)	40.2 (21-56)	43.1 (22-80)
Diagnosis	CIS with MRI lesions (3)	IIH (6)
	RRMS (9)	Small vessel disease (4)
	SPMS (1)	Cord lesion (3)
	PPMS (3)	Functional (2)
		MND/ALS (1)
		Other (7)
Number of samples with detectable CSF lymphocytes (%)	7 (44%)	4 (17%)
Number of subjects with detectable CRP (>5mg/l) (%)	1 (6%)	5 (22%)
Median number of T2 lesions on MRI (range)	5 (2->10)	N/A
Median number of gadolinium enhancing lesions (range)	1 (0-5)	N/A

3.2.3. Laboratory techniques

CSF samples were assessed for the presence of oligoclonal bands in the immunology department at the Royal London Hospital using IEF with immunofixation (figure 2.1, page 19). κ and λ FLC levels were assessed in CSF and urine using a commercially available ELISA (BioVendor, Brno Czech Republic). The assay was carried out to the manufacturers' instructions, with CSF diluted 1:10 prior to the assay. Neopterin levels were measured using a commercially available ELISA (IBL, Hamburg, Germany) according to the manufacturer's instructions. Samples had not been exposed to light for prolonged periods, and the assay was performed in the dark. UCHL1 levels were measured using a commercially available ELISA (USCN, Wuhan China) without prior dilution of samples.

Urinary protein and creatinine were measured using commercially available kits (protein: SigmaAldrich, St Louis MO USA; creatinine: R+D Systems, Minneapolis USA). These assays were performed according to the manufacturers' instructions. Urinary FLC levels were expressed as a ratio to total protein, and neopterin and UCHL1 as a ratio to creatinine in order to correct for urine concentration and glomerular protein loss.

Protein was used as the calibrator for FLC primarily because FLC are themselves proteins. FLC are renally excreted as 22kDa monomers, 44kDa dimers, as fragments or as multimers – this can be unpredictable in individual patients (81). Whilst the immunoassay has been designed to measure all of these eventualities, the forms excreted will depend on the glomerular filtration to various protein sizes in a given individual. This variation is controlled for by measuring total protein – if an individual has heavy proteinuria they would be expected to excrete increased FLC overall compared to an individual with normal glomerular filtration. In contrast, both neopterin and UCHL1 are small molecules which would be expected to be freely filtered at the glomerulus. This is much more akin to the filtration of creatinine, which is a single small molecule, hence this is used to correct for concentration

rather than total protein which represents a combination of concentration and glomerular glomerular protein loss.

3.2.4. Statistical analysis

Statistical analysis was performed using PASW v18 (SPSS). Variables were tested for normality using a Shapiro-Wilk test. Variables which were not normally distributed were normalised using a natural log transformation. Correlations were evaluated using Pearson coefficient, and differences between OCB positive and negative groups using an unpaired t-test. The p-value for significance (α) was set at 0.05. A conservative analysis was performed using a Bonferroni correction, with α=0.01.

3.3. Results

Mean values for each biomarker are shown in table 3.2.

Table 3.2: mean values of CSF and urinary biomarkers

	OCB positive (mean (SD))	OCB negative (mean (SD))
CSF protein (mg/l)	391.3 (159.2)	480.0 (170.2)
CSF IgG (mg/l)	67.9 (61.1)	33.1 (16.5)
CSF total FLC (ug/l)	1337.0 (1576.5)	189.8 (59.8)
CSF kappa FLC (ug/l)	980.2 (1103.6)	72.0 (42.7)
CSF lambda FLC (ug/l)	356.9 (664.7)	117.9 (24.1)
Urine total FLC:protein (ug/l:mg/dL)	7420.0 (8015.7)	13547.5 (16578.8)
Urine kappa FLC:protein (ug/l:mg/dL)	5311.1 (6898.8)	10403.0 (14235.1)
Urine lambda FLC:protein (ug/l:mg/dL)	1411.5 (1166.0)	2963.4 (3632.1)
CSF neopterin (nmol/l)	9.49 (19.94)	32.14 (20.00)
Urinary neopterin:creatinine (nmol/l:mg/dL)	23.7 (13.33)	23.94 (52.89)
CSF UCHL1 (pg/ml)	No samples positive	38 (41.8)[1]
Urine UCHL1:creatinine (pg/ml:mg/dL)	1.30 (1.12)[2]	0.67 (0.34)[3]

[1] only 3 positive samples
[2] only 8/16 samples positive
[3] only 10/23 samples positive

3.3.1. CSF and urine free light chains

CSF FLC were higher in the MS group (p<0.001) (Figure 3.1a). There was no difference in urinary FLC between the MS and control groups (Figure 3.1b), and no relationship between CSF and urinary FLC levels (Figure 3.2). When κ and λ FLC were studied, the relationship between CSF OCB positivity and FLC remained.

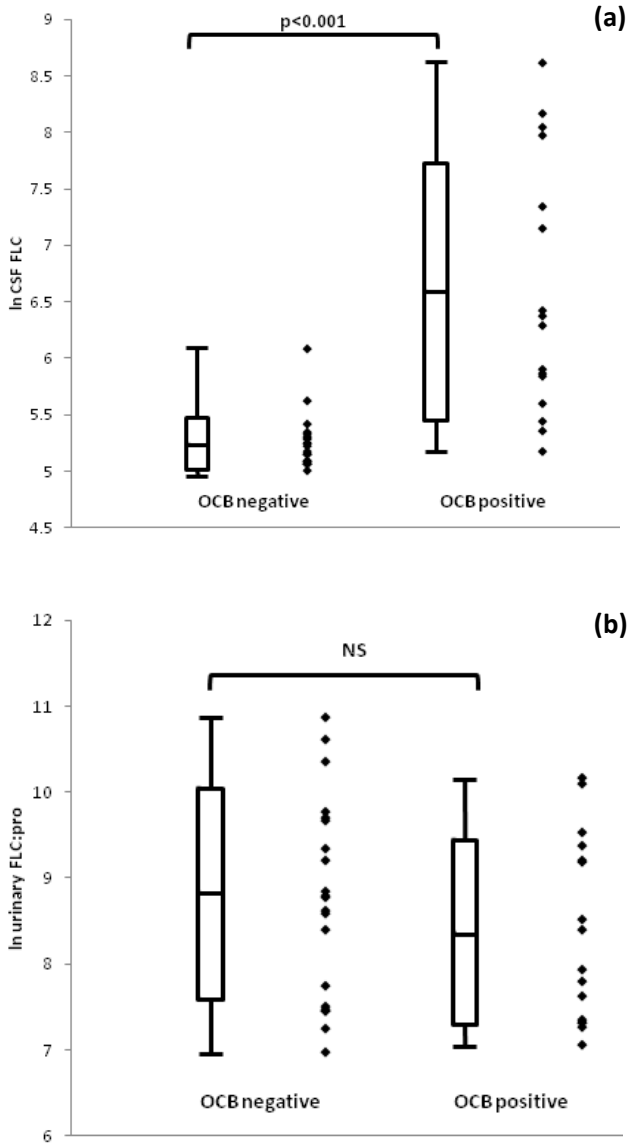


Figure 3.1: (a) Combined box and whisker and scatter plot demonstrating that CSF FLC are higher in those who are OCB positive. The box indicates the mean and standard deviation for each group, and the whiskers the range. **(b)** Combined box and whisker and scatter plot demonstrating no difference in urinary FLC levels between OCB positive and OCB negative patients. The box indicates the mean and standard deviation, and the whiskers the range.

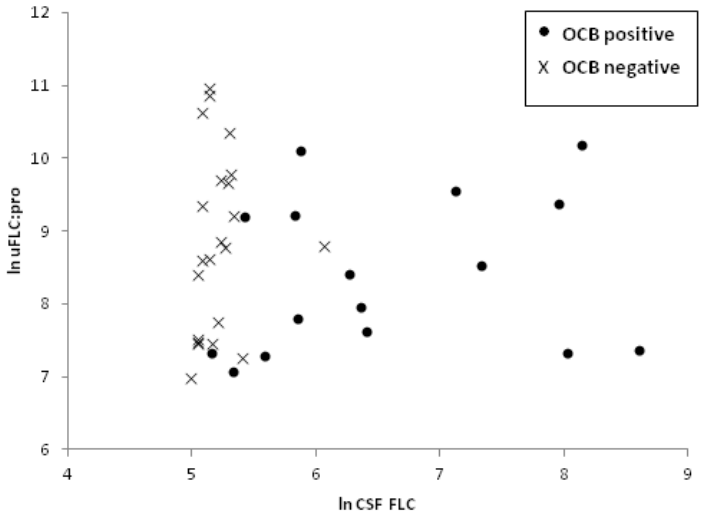


Figure 3.2: No relationship between CSF and urinary FLC levels. When only OCB positive cases were selected, again no relationship was seen.

3.3.2. Relationship between CSF biomarkers

There was a significant correlation between total CSF FLC and CSF neopterin in MS samples (correlation coefficient=0.588, p=0.016; borderline significance on conservative testing; Figure 3.3a). There was a strong correlation between CSF λ FLC and CSF neopterin in MS samples (correlation coefficient=0.875, p<0.001, Figure 3.3b), which was maintained with conservative α; however when the outlying point was removed significance was lost. There was no relationship between CSF total, κ, or λ FLC and CSF neopterin when all samples were included in the analysis.

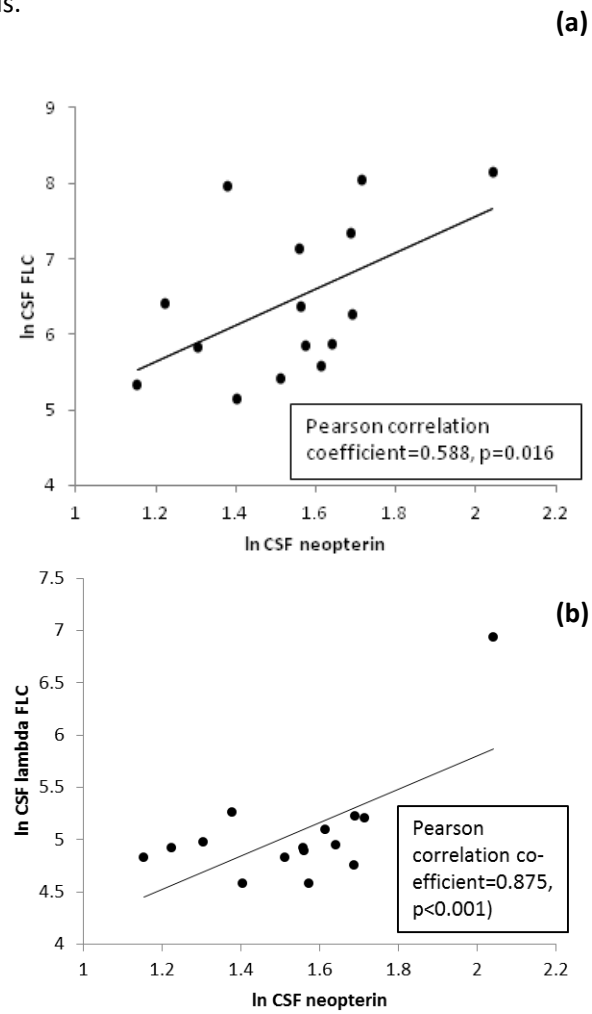


Figure 3.3: (a) There is a strong relationship between CSF neopterin and CSF FLC levels in the MS group, Pearson Correlation co-efficient=0.588. **(b)** There is a relationship between CSF neopterin and CSF λ FLC levels in the MS group, Pearson Correlation co-efficient=0.875; this is lost when the outlier is removed.

3.3.3. Relationship between urinary biomarkers

There was a strong significant correlation, maintained on conservative testing, between urinary neopterin:creatinine levels and urinary total FLC:protein levels (correlation coefficient=0.452, p=0.004, Figure 3.4). This relationship was also seen with urinary λ FLC:protein (correlation coefficient=0.419, p=0.009). The relationship between urinary neopterin:creatinine and urinary λ FLC:protein was borderline when MS samples were selected (correlation coefficient=0.555, p=0.026).

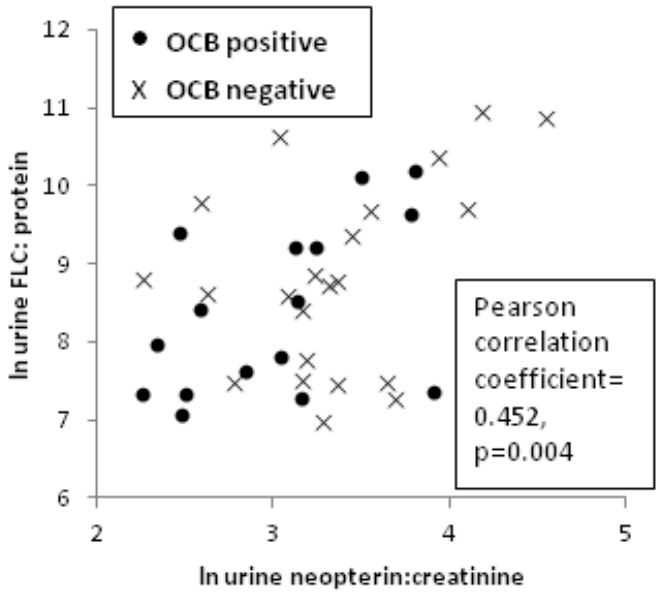


Figure 3.4: There is a strong relationship between urine neopterin:creatinine and urine total FLC:protein levels when looking at all samples (Pearson Correlation co-efficient=0.452). However this is lost when only the OCB positive samples are selected, possibly as a result of small numbers.

3.3.4. CSF and urinary UCHL1

Only 3 CSF samples (8%) had detectable levels of UCHL1. 18/38 (48%) (8/15 MS and 10/23 control) urine samples had detectable levels of UCLH1. It was therefore not possible to determine a relationship between CSF and urine UCHL1. There was no difference in urinary UCHL1 levels between MS and control samples.

3.3.5. CSF and urinary neopterin

There was no difference in CSF or urinary neopterin between the MS and control groups (data not shown). There was no correlation between CSF and urinary neopterin overall, although when the MS cases were selected there was a trend towards a significant relationship between CSF and urine neopterin (correlation coefficient=0.545, p=0.029, Fig 3.5); this was not significant on conservative analysis.

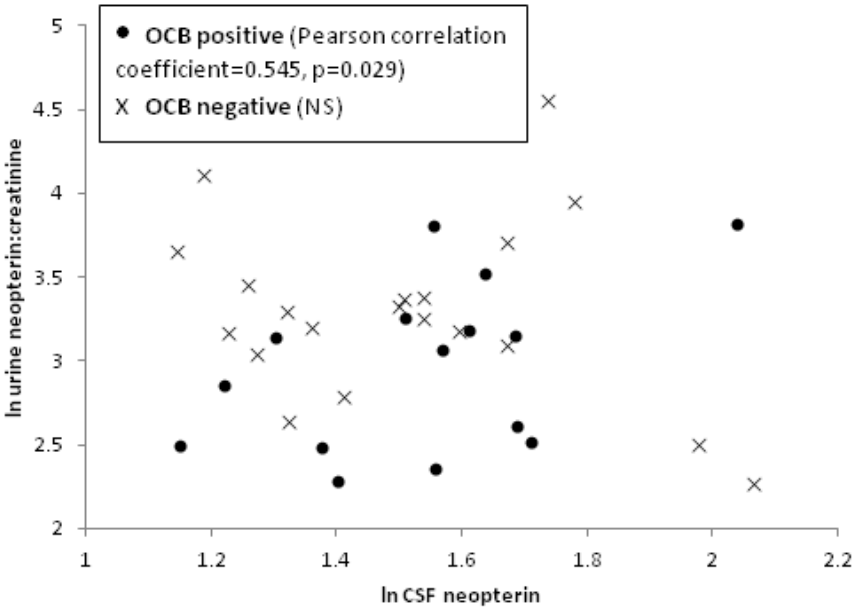


Figure 3.5: No relationship between CSF and urinary neopterin levels. When only OCB positive cases were selected, no relationship was seen.

3.3.6. Relationship between CSF and urinary biomarkers

There was no significant correlation between CSF FLC and urine neopterin:creatinine or between CSF neopterin and urinary FLC:protein.

3.4. Conclusions

This study confirms the relationship between CSF OCBs and CSF FLC, indicating the importance of intrathecal B- and plasma cell activation in MS. There is a relationship between CSF FLC and CSF neopterin in MS, highlighting the multi-faceted immune activation seen in MS. Although urinary FLC correlate with urinary neopterin, this relationship is lost in subgroup analysis of the MS samples, probably as a result of the small number of samples in this group. I was not able to confirm an increase in urinary FLC in the OCB positive group (41), indicating that urinary FLC are not a suitable surrogate marker for CSF OCBs. This is potentially due to the large difference in the concentrations of FLC between the CSF and the urine, indicating a significant potential dilutional effect during FLC excretion. The relationship between CSF and urinary neopterin levels in MS was not significant when a Bonferroni correction was applied.

Both CSF FLC and CSF neopterin have previously been shown to be increased in MS; however, no correlation between the levels of these biomarkers has previously been demonstrated. The implication of this finding is that those patients with higher levels of B- and plasma-cell activity in MS also have higher levels of T-cell activation. This finding may reflect disease activity in individual patients, or possibly reflect disease stage; however this study was not of sufficient power to examine this.

The low levels of CSF UCHL1 seen may reflect the fact that the patients in this study have relatively early stage disease. UCHL1 correlates with NFh in sub-arachnoid haemorrhage (72), and levels of NFh are increased in late stage RRMS and SPMS, reflecting chronic axonal degeneration (82). Elevated levels of tau and s100b have been detected in the CSF and serum of people with MS, providing further serological evidence of axonal pathology (83). However, no attempt has been made to study the relationship between UCHL1 and NFl, tau

or s100b, either in CSF or serum. Given the high molecular weight of neurofilament (NFl 68kDa and NFh 190-210 kDa), it is not likely to be filtered at the glomerulus and therefore urinary levels are unlikely to be useful. The control group was a rather heterogeneous group, all of whom were undergoing a lumbar puncture for diagnostic and/or therapeutic purposes. A relatively large proportion of these patients (6/23) had a diagnosis of idiopathic intracranial hypertension, IIH. The advantage to using these patients is that there is often a large volume of CSF available for research use. However, it has been documented that they have increased levels of cytokines both in the CSF and serum, including CCL2 in the CSF and CCL7, CCL8 and interleukin1-α in the serum (84). Although neopterin has not been previously studied in this patient group, it may be that neopterin levels are increased in IIH.

The finding that the control patients in this study had higher than expected urinary FLC and neopterin suggests that many of them may have an on-going low-grade inflammatory response. The mean CSF protein level was elevated in the control group at 480mg/l, with 4 samples containing lymphocytes; 22% control subjects had raised serum C-reactive protein. Haptoglobin levels have been shown to be increased in the CSF of patients with both chronic inflammatory demyelinating polyneuropathy (CIDP; included in the control group for this study) and MS but not in Guillian-Barre syndrome or other neurological diseases (85), highlighting the CSF changes that may confound studies such as this. Whilst this study was intended to be a pragmatic study, examining the utility of potential biomarkers for use in the diagnosis of MS, there remains a need for consensus on control groups used, as these vary between individual centres and studies.

There is a need for further studies to examine the relationship between both CSF and urine FLC and neopterin, in order to better understand the relationship between these biomarkers in the context of MS. The hypothesis that the combination of FLC and neopterin levels may reflect disease activity needs to be studied in more detail. It should also be determined

whether urine neopterin can be used as a surrogate for CSF neopterin in a larger cohort, as if neopterin provides a useful reflection of disease activity, then monitoring urinary levels will be of clear interest, as previously discussed.

The use of urinary biomarkers in MS therefore demands further attention in order to both develop biomarkers for practical use in MS and better our understanding of MS pathogenesis. Whilst the evidence produced in this small-scale study does not support the contribution of urinary biomarkers to a putative risk score in MS, they will be further evaluated in the context of this, and the results presented later in this thesis.

Chapter 4: Endophenotype study

4.1. Background

4.1.1. Defining an endophenotype

Despite extensive research efforts, much remains to be understood about the mechanisms underlying the initiation of MS. It is unlikely that MS is the result of a single causative event; instead the disease develops in a genetically susceptible population as a result of environmental exposures.

The concept of a prodrome is being studied intensively in a variety of neurological diseases. It is defined as the duration between the onset of decline in a baseline level of functioning until the time at which the criteria for the diagnosis of a disease are met (86). The constellation of symptoms in a prodrome tend to be nonspecific, especially in the early stages (86). This concept overlaps with that of the endophenotype, where the disease spectrum is extended to those at risk of disease development, allowing the study of the trajectory of changes in the disease process from genetic risk factors to clinical diagnosis (86). The endophenotype is associated with illness in the population, is more common in family members and manifests in an individual whether or not disease is active (87). It can take a variety of forms, and may be thought of as a disease "trait" in the broadest sense of the word. Those at higher risk of demonstrating the disease endophenotype are those carrying genetic risk factors for a given disease, such as first degree relatives of patients with the disease.

Can the concept of an endophenotype be applied to MS? The diagnosis of MS remains a clinical one, supported by paraclinical findings, and confirmed after the exclusion of other diseases (88). The majority of patients with MS initially present with a CIS, defined as a distinct first neurological event of demyelination which can potentially affect any part of the CNS (89). However, radiological abnormalities are increasingly being identified in the

absence of clinical symptoms, which has led to the development of the label "radiologically isolated syndrome" (RIS) (90). Individuals with a RIS are at increased risk of developing MS, although the magnitude of this risk has yet to be accurately quantified. As the frequency of MRI scanning increases, RIS is becoming an increasingly common finding, most commonly in the context of MRI being performed for the investigation of headache syndromes. Some patients who are thought to have a RIS show subclinical cognitive impairment in keeping with that seen in early MS (91), highlighting the potential prodromal nature of the condition. Approximately two-thirds of persons with RIS show radiological progression and one-third develop neurological symptoms during mean follow-up times of up to five years (91). A further feature in support of an MS endophenotype is the fact that MS is more common among members of the same family. Even if siblings of people do not meet the diagnostic criteria for MS, they often display clinically silent changes associated with MS (92, 93).

Further possible endophenotypic changes that may be seen in the context of MS include CSF oligoclonal bands, and peripheral markers of immune abnormalities that have previously bee associated with MS. However, whilst many such changes have been studied in people with MS compared to healthy controls, their study as part of an endophenotypic construct has not been performed to date. Through the development of a tool to calculate MS risk, based on existing evidence regarding MS risk factors, I hope to be able to identify those unaffected siblings with a higher probability of demonstrating changes in keeping with the MS endophenotype.

The above characteristics underpin our understanding that changes in the CNS very similar to those eventually causing the symptoms of MS develop over a number of years prior to the initial clinical presentation. An excellent illustration of this can be seen in patients who at first presentation have a large disease burden on MRI already; clearly this lesion load must have built up over months or years prior to the emergence of clinical symptoms. Thus,

the concept of an endophenotype in MS has much to offer in terms of our understanding of disease pathogenesis and development. Through the study of the pathways leading to the first clinical manifestations of MS, I hope to better understand the causal cascade and hopefully inform future prevention studies.

4.1.2. Risk factors in multiple sclerosis

4.1.2.1. Family history

There is considerable evidence that MS occurs in a genetically susceptible population. The importance of genetic factors in susceptibility to MS has been demonstrated by genetic and epidemiological studies (94). Studies assessing the risk of MS in relatives of MS probands have revealed a marked familial aggregation of the disease. First degree relatives generally have a 10-25 times greater risk of developing MS compared to the rest of the population. The increase in risk correlates with the degree of kinship (95, 96) (figure 4.1) with parent-of-origin effects (see below) and gender influencing the size of these risks (96, 97).

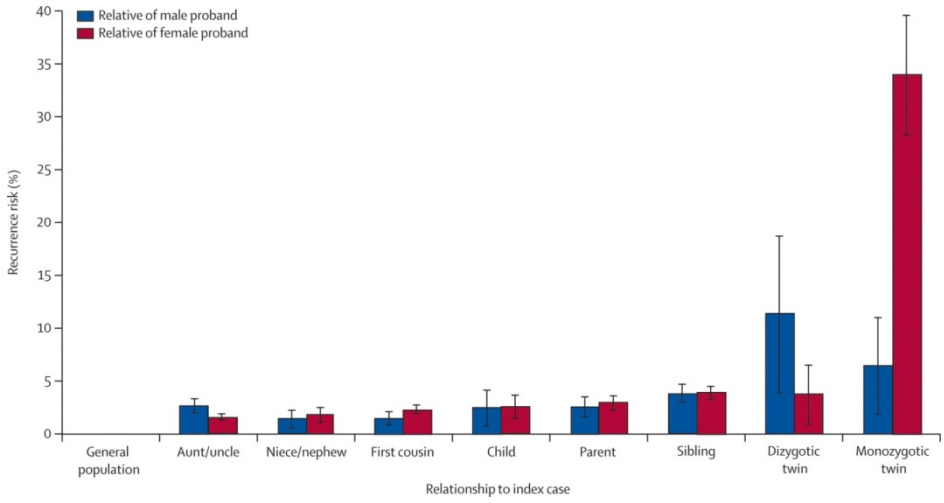


Figure 4.1: Age-adjusted percentage recurrence risks for relatives of multiple sclerosis probands. Data are mean with bars indicating standard error. Taken from Ramagopalan 2010 (2).

In individuals related to a person with MS, the risk of MS appears to be modulated by not only the strength of the genetic relationship, but also the nature of this relationship – the "parent of origin" effect. Compared to maternal half-siblings, paternal half-siblings of

patients with MS appear to have a significantly reduced risk of MS (2.35% vs. 1.31% respectively), whereas in full siblings the risk is comparable (2.35% vs. 3.11%) (98). The mechanisms underlying this apparent differential transmission of risk remain speculative, but are likely to involve epigenetic mechanisms (99).

4.1.2.2. Genetic basis of MS

Multiple sclerosis (MS) does not behave as an inherited disease in the Mendelian sense, but there is a wealth of evidence supporting the role of genetic factors in influencing disease susceptibility. The first gene associated with MS susceptibility, the major histocompatibility complex (MHC) allele HLA-DRB1*1501, was discovered in the early 1970s using a candidate gene approach. This gene confers a relative risk of MS among heterozygotes of between 2 and 3 (6.7 in homozygotes) (100). Despite scores of subsequent candidate gene studies, no other genetic locus for MS was conclusively identified using this technique, and there remained much to be learnt about the genetic basis of MS. Extensive studies have shown that other HLA haplotypes are both positively and negatively associated with disease risk, differ in magnitude of effect, and interact with one another (figure 4.2).

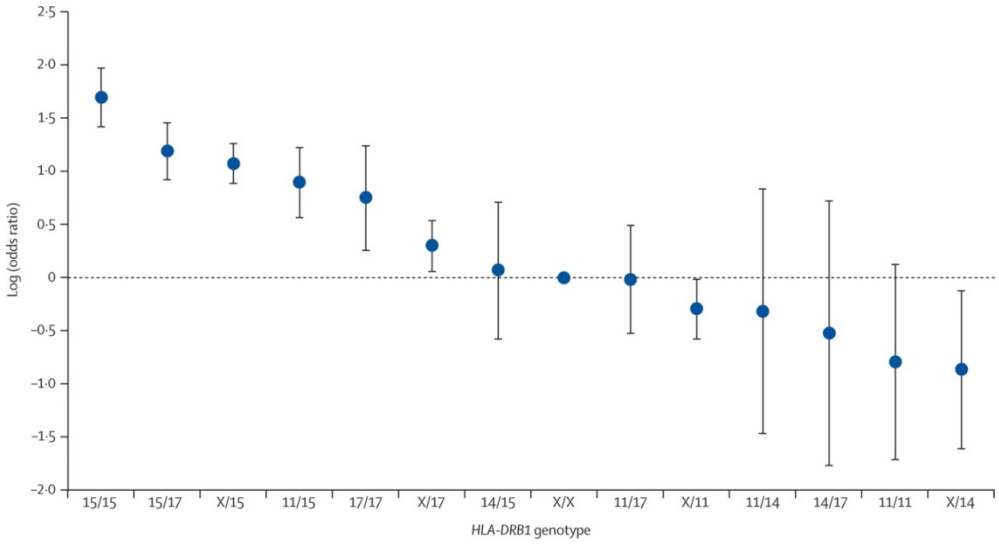


Figure 4.2: HLA interaction in MS: genotypic odds ratios for multiple sclerosis for combinations of alleles at the *HLA-DRB1* locus.
X/X=individual with no disease-associated alleles; baseline odds ratio of 1·0 (dotted line).
X=any non-disease-associated allele. Numbers (01, 08, 10, 11, 14, 15, 17) indicate HLA-DRB1 alleles associated with multiple sclerosis.
Figure taken from Ramagopalan 2010 (2).

The past 10 years have seen a revolution in the field of genetics – from the first complete sequencing of the human genome in 2003 to more recent large-scale genome-wide association studies (GWAS). GWAS involve genotyping thousands of single nucleotide polymorphisms (SNPs) across the genome in hundreds (or thousands) of subjects with a given disease, comparing them to matched controls. Through this technology, the entire genome can be scanned simultaneously, and genetic variants influencing disease risk established.

The first GWAS in MS was performed in 2007. The International Multiple Sclerosis Genetics Consortium published the results of an international collaboration identifying 17 SNPs strongly associated with MS (101), with further data generated in the same year by the Wellcome Trust Case Control Consortium (102). Outside of the HLA-DRB1*1501 allele, the relative risks conferred by the risk SNPs identified were relatively modest, with odds ratios (OR) in the region of 1.1-1.3 (101). A follow-up GWAS in 2011 (103) built on this, validating 23 of the non-MHC SNPs identified in the original GWAS as contributing to MS risk, identifying a further 29 non-MHC SNPs as significant additional contributors, and finding strong associations between five additional SNPs and MS.

Additional studies have identified rare mutations with larger effect sizes (104). These rare, low frequency variants are not present on current SNP genotyping arrays, meaning that they are not detected by large-scale GWAS. It is becoming increasingly clear that so-called rare variants are in fact relatively common (105), suggesting that individual disease risk may be influenced by rare or indeed private (confined to one individual/family) mutations. Examples of these in MS have recently been described and it is extremely likely that more will follow (104, 106). However, to date, these findings have not been replicated (107, 108). There are a number of possible explanations for the lack of replication; firstly, the initial study could have been a false-positive result, or the effect seen may be a population-specific effect

specific to the Canadian population. Discovering such rare variants with large effect sizes will require sequencing of genomes in large patient numbers. Whilst this is rapidly becoming affordable, the yield is highly dependent on the minor allele frequency, which is the primary unknown variable.

It is possible that epigenetic changes explain some of the discordance in MS seen in genetically identical monozygotic twins. If GWAS were fully able to predict MS susceptibility, MZ twin concordance rates would approach 100%; however, they are around 25% (3). Outside of MS, epigenetic differences between MZ twins have been demonstrated (109). To date, only one study has examined methylation status in MZ twins discordant for MS (110), and this did not uncover any differences. However, this study had an extremely small sample size (n=3), and this, together with the high stringency measures, mean that there is more work to be done in this arena. Epigenetic differences will undoubtedly add to risk prediction once they are uncovered, and this is an area that is rapidly expanding at the present time.

4.1.2.3. Sex

MS is more common in females than in males, with an estimated relative risk (RR) of 2.62 (111). A similar influence of sex is apparent in both CIS and RIS (112), indicating that sex-specific factors play a role in MS disease development at an early stage. However, GWAS have failed to provide any convincing support for MS risk being conferred by genes present on the X-chromosome; it has been suggested that this sex ratio may be related to female specific physiology (113). There is an increasing incidence of MS in females relative to males (114), which remains unexplained.

An interesting observation has been the increasing sex ratio of MS over the last century (37, 114, 115) (figure 4.3). Large population-based studies in Canada and Denmark have clearly demonstrated this phenomenon (37, 114); however they have not been able to shed any light on the underlying aetiology of this change. This change in the sex ratio has not been demonstrated in all countries with such large-scale population registries – a recent study originating from Sweden demonstrated a remarkably stable sex ratio over time (111). Conversely, a dramatic change in the sex ratio of MS has been seen in Iran, with an exponential increase in MS prevalence in females compared to a relatively stable prevalence in males (116).

The potential reasons underlying the increase in prevalence in females seen in certain countries remain opaque. Much has been made of possible trends involving decreasing serum vitamin D levels in women, in the context increased sunscreen use and changing attitudes towards sunbathing (117). Most notably, this has been described in Iran with the widespread adoption of the veil following the 1979 revolution (118). However, there may be other reasons underlying this change, which after all is not universal.

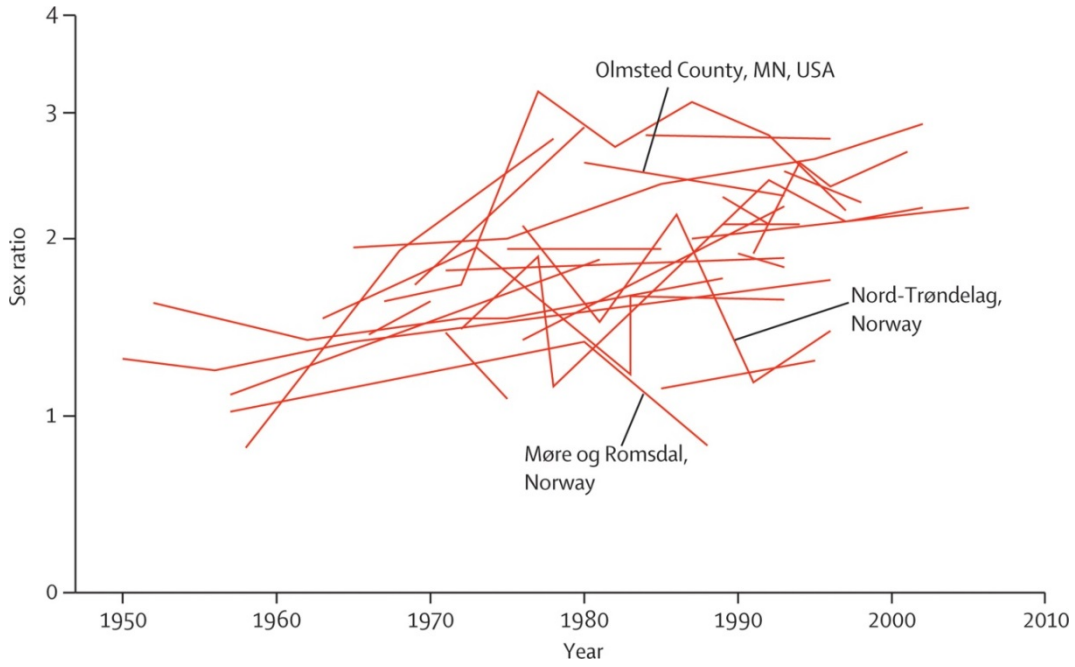


Figure 4.3: Change in female:male sex ratio of MS. Sex ratios are shown on a logarithmic scale. Figure taken from Koch-Henriksen 2010 (37).

One theory has been that changes in the sex ratio of smoking, which mirrors that seen in MS, could be responsible. However, whilst the changing sex ratio in MS is primarily driven by increasing rates of MS in women, the change in smoking ratio is primarily driven by decreasing rates of smoking in men (119). It may be that there were previously differential access to healthcare services between males and females, with males more likely to present to healthcare systems (120), and this difference may have been especially marked with respect to the often non-specific symptoms seen in MS. With the changing diagnostic criteria for MS, the relapse rate appears to have fallen, although whether this is an effect of increased rates of diagnosis, often at an earlier stage, remains to be seen (the "Will Rogers effect", where observations are dependent on the population in which they are made).

4.1.2.4. Latitude

Within regions of temperate climate, MS incidence and prevalence increases as latitude increases (121). A recent meta-analysis demonstrated a significant but weak association between prevalence and latitude (figure 4.4) (r^2=0.045; p=0.018; regression coefficient 1.29 per 100,000 per degree latitude) (37). More complex patterns of disease distribution do however exist. In Norway for example, MS prevalence does not increase with latitude: prevalence here correlates with proximity to coastal fishing areas and subsequent fish consumption (122). Some of the geographical distribution of MS can be explained on the basis of ethnicity and genetic factors (123) but latitude remains the strongest geographical factor for risk after controlling for ethnicity (124).

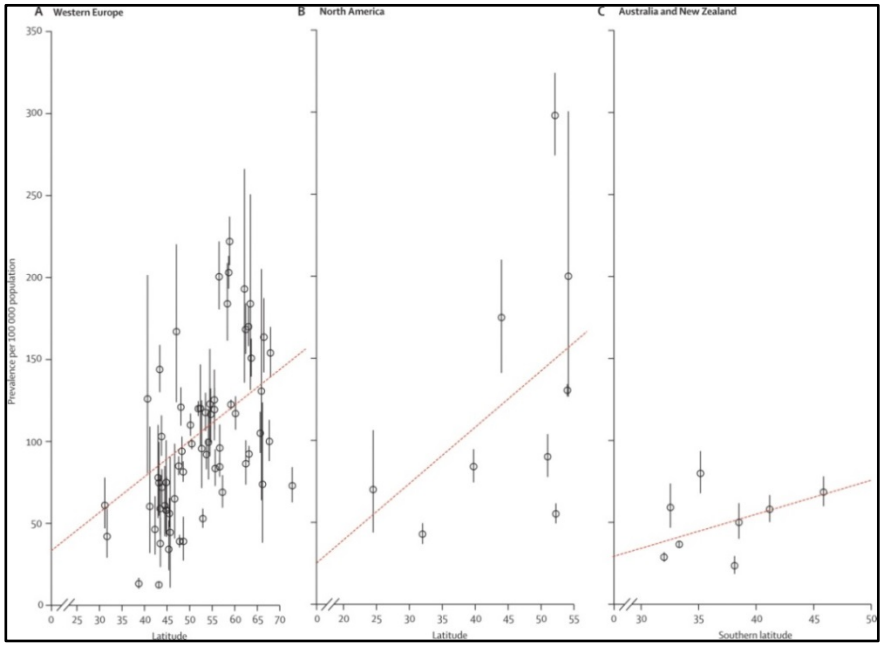


Figure 4.4: MS prevalence by geographical latitude. Taken from Koch-Henriksen 2010 (37)

The effects of migration between high and low risk geographic regions for MS has been examined in several populations. These studies consistently show that MS risk is influenced

at least to some extent by country of origin (125). Despite the limits of small sample sizes, a 'critical age' has been hypothesized: immigrants who migrate before adolescence acquire the risk of their new country, while those who migrate after retain the risk of their home country (126). The influence of place of birth is highlighted by the fact that first generation Afro-Caribbean and Asian immigrants to Britain have a much lower incidence of MS than their second generation counterparts born in the UK (127).

4.1.2.5. Month of birth

Month of birth has also been shown to affect MS risk in a number of studies. A recent meta-analysis pooled all of the available data and demonstrated a significant excess of MS risk in those born in April (observed:expected ratio 1.05, p=0.05), and a reduction in risk in those born in October (O:E 0.95, p=0.04) and November (O:E 0.92 p=0.01). A conservative analysis of 78,488 patients revealed an excess MS risk in those born in April (O:E 1.07, p=0.002) and May (O:E 1.11, p=0.0006), and a reduced risk in those born in October (O:E 0.94, p=0.004) and November (O:E 0.88, p=0.0002) (128).

4.1.2.6. Epstein-Barr virus infection

Virtually all people with MS (>99%) have evidence of prior infection with EBV compared to ≈94% of age-matched control subjects (129). The corollary to this is that MS is very rare in adults who have not been infected with EBV; the relative risk of MS in an EBV negative individual is very low (OR=0.07; 95%CI 0.03-0.16) (130). Furthermore, MS has been observed to occur only after EBV infection, as demonstrated during longitudinal follow-up of a large cohort of EBV-negative young adults (131). People with high titres of anti-EBV antibodies have a higher risk of developing MS compared to subjects with low titres (132, 133). There appears to be a temporal relationship – plasma antibody titres against the EBV nuclear antigen-1 (EBNA-1) increase several years prior to the clinical onset of MS (132, 133). The risk of MS associated with the presence of anti-EBNA-1 IgG antibodies is dependent on the technique used for antibody detection – indirect immunofluorescence has a far higher sensitivity for antibody detection than the more widely-used ELISA technique (130).

Further supporting a role for EBV in MS is the finding that individuals with a history of infectious mononucleosis (IM) have an increased risk of developing MS. A systematic review and meta-analysis of 14 case-control and cohort studies reported a combined conservative relative risk of MS after IM of 2.3 (95% CI 1.7-3.0) (134). This risk has subsequently been confirmed in large population based studies (135, 136).

Whether or not EBV genotype influences MS risk remains an unanswered question. A study of genetic variability in EBV strains between MS patients and controls did not provide evidence of specific EBV strains associated with MS, although it did show differing frequencies of single nucleotide polymorphisms in the EBNA-1 and BRRF2 genes (137). EBV can be typed as type 1 or type 2, according to polymorphisms in the EBNA2 gene. Recent data has indicated a possible relationship between MS and the presence of dual infection

with EBV 1 and 2; however, this is descriptive evidence and mechanistic insights linking co-infection with the pathogenesis of MS are still lacking (138).

4.1.2.7. Vitamin D

Sunlight exposure and associated serum 25-hydroxyvitamin D levels represent a possible explanation for the link between latitude and the risk of MS (139). Levels of past sun exposure are inversely related to MS susceptibility (adjusted OR for high summer sun exposure (2-3 hours per day) during childhood and adolescence=0.31 (95% CI 0.16-0.59) (140)). Questionnaire based studies are prone to recall bias, but confirmation of an effect of sun exposure on MS risk was seen when examining actinic damage, an objective measure of sun exposure. Greater actinic damage is associated with a decreased risk of MS (OR=0.32, 95% CI=0.11 to 0.88 for grades 4-6 of damage), however the timing of damage could not be accurately determined in this retrospective study. Additional evidence has demonstrated that the gradient in MS prevalence in France closely matches the UV exposure index gradient (141) (figure 4.5); this phenomenon can also be demonstrated in England, despite the small size of the country (142).

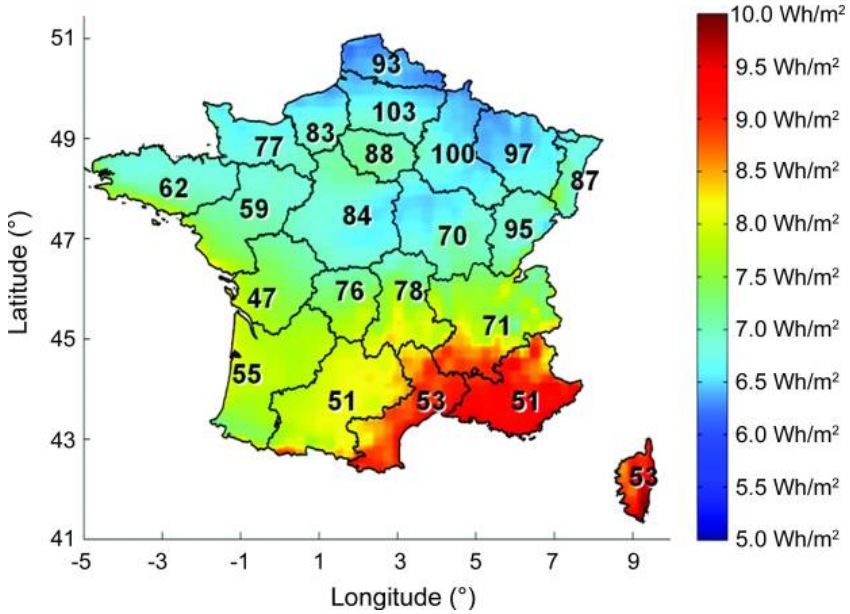


Figure 4.5: Combined figure showing annual mean ultraviolet B (Wh/m^2) radiation in France together with multiple sclerosis prevalence rates (per 100,000) for each Mutualité Sociale Agricole region. Figure taken from Orton et al (141).

Experimental and epidemiological data suggest that vitamin D is the mediator of the sunlight effect. It was noted many years ago that the consumption of fatty seafood and cod liver oil in Norway, both rich sources of vitamin D, provided protection against the risk of MS (122). A prospective cohort study found that taking vitamin supplementation which included vitamin D was associated with an approximate 40% reduction in the risk of developing MS (143), but the amounts of vitamin D taken are thought to be insufficient to significantly alter circulating vitamin D levels (144). Additionally, the effects of multivitamin intake may be confounded by behavioural differences. There is some evidence that UV exposure may have a protective effect on overall MS risk independent of serum 25-hydroxyvitamin D levels (145, 146), and these findings deserve further attention.

The best evidence for a role for vitamin D comes from a prospective, nested case-control study of military personnel in the United States with stored serum samples. This showed that a lower risk of MS was associated with high serum 25-hydroxyvitamin D levels at enrolment (147). It may be that there is an additional link between vitamin D levels and MS relapses. Vitamin D levels have been inversely linked to MRI markers of MS activity (148), and this is supported by a similar relationship between vitamin D levels and relapse rate (149). However, consumption of vitamin D during immune activation seen in relapses may provide an explanation for this, in terms of reverse causality (150). A large meta-analysis found an excess of hospital events related to MS in the spring months, with a nadir in winter (151), and a similar pattern has been found in hospital admissions in Scotland (152). This pattern has not been explained to date, but it has been argued that this seasonality may be related to fluctuations in vitamin D levels (153).

4.1.2.8. Smoking

It has been known for some years that smoking appears to increase MS risk. A recent meta-analysis indicated that the risk of MS was higher in smokers than non-smokers (RR 1.54; 95% CI 1.41-1.69, $p<10^{-21}$) (154). The effect of smoking on risk of progression to secondary progressive MS was less clear (RR 1.88; 95%CI 0.98-3.61, p=0.06) (154), however few studies have addressed this. Interestingly, Swedish snuff use does not appear to increase the risk of MS (OR=0.3, 95% CI=0.1–0.8), suggesting factors present in smoked tobacco or the route of administration as being important (155).

4.1.3. Risk factor interaction in MS

Recently, attention has shifted towards studying the interaction between MS risk factors. These studies have yielded considerable effect sizes, with MS risk factors appearing to act synergistically. In those patients who have already developed a CIS, the presence of OCBs and MRI lesions compatible with demyelination are strongly predictive of the development of clinically definite MS (34). Conversely, in this study, the 25 subjects with a normal MRI and no OCBs did not go on to develop MS (34).

The interaction between specific risk factors measured in this study will be discussed in more detail in section 4.3.3. However, one point that deserves particular attention is the potential for gene-environment interactions in MS susceptibility, which are poorly typified using current techniques. Gene-environment interactions almost certainly play a role in MS susceptibility, but are missed by GWAS. The effect of gene-environment interactions on the strength of genetic contribution to disease is difficult to estimate outside studies examining specific relationships between defined genetic markers and environmental exposures. Additionally, the population size that would need to be studied to examine the interaction between two risk factors, both of which confer a relatively small (i.e. OR in the region of 1.2) effect on disease risk requires prohibitively large studies. It has not proved possible to overcome this significant limitation to date, with relatively small scale and thus underpowered studies providing the only evidence from which conclusions must be drawn with caution.

The influence of gene-environment interactions has been demonstrated in MS. IgG directed against specific EBV fragments has been demonstrated to interact with both the presence of HLA-DRB1*1501 and the absence of HLA-A*02 to adjust MS risk (156). The inclusion of environmental factors has been shown to improve a putative MS risk score – when 16

genetic variants were considered in isolation the area under a receiver operating characteristic curve (ROC curve) was 0.64; including gender in the model improved the area under the curve (AUC) to 0.72 and the inclusion of both smoking and IgG titres against an EBNA-1, an EBV antigen, further improved the AUC from 0.64 to 0.68 (157). Similarly, gene-gene interactions may be modified by environmental factors– the interaction between the HLA-DRB1*1501 risk allele and the absence of the HLA-A*02 protective allele is only significant in smokers (OR of MS in smokers 13.5); however in non-smokers the interaction disappears and the OR is a more modest 4.9 (158).

The environment clearly has a highly influential role in the development of MS, and this must be considered in terms of both exposure and potential influence on pre-existing genetic risk when modelling MS susceptibility. The ultimate aim is not necessarily to predict an individual's risk but to identify high-risk cohorts that will enable the MS community to design and test prevention trials that will economically viable and read-out in a reasonable period of time. Whilst this study is not designed or able to measure MS susceptibility in terms of disease development over longitudinal follow up, it is able to provide an indication of the accuracy of a putative MS risk score with the use of both immunological and MRI markers of pre-clinical MS disease activity.

4.1.4. Immune abnormalities in MS

4.1.4.1. T-cell findings

The longstanding dogma is that MS is primarily a CD4+ T-cell mediated disease (159). Different subpopulations of CD4+ T cells with a characteristic cytokine profile exist, namely pro-inflammatory T_H1, T helper T_H2 and T_H17 cells (159). It was traditionally thought that T_H1 cells were the prime drivers of the autoimmune process in MS. However, treatments specifically targeting CD4+ T cells in MS are ineffective (160), although this may have been because the level of T-cell depletion was insufficient.

Interleukin-17 (IL-17) producing T_H17 cells, a recently identified lineage of CD4+ T cells, have been shown to play a role in MS pathogenesis. IL-17 levels are increased in patients with MS (161, 162), rise during relapse periods (163), and fall with treatment (161, 164). IL-17F levels are a potential predictor of interferon treatment response in MS (165), although this finding has not been replicated (166). Myelin-reactive T_H17 cells are able to efficiently secrete cytokines and induce CNS inflammation in EAE, the animal model of MS (167). IL-17 levels are higher in early MS (duration <2 years) than later disease (168). IL-17 stimulates macrophages to produce IL-6, TNF-α and IL-1β, which have been implicated in MS pathogenesis (169).

Another subpopulation of CD4+ cells, CD4+CD25^{high} T cells (T_{regs}) have been shown to play a role in immune homeostasis in MS (170). The subgroup of CD4+/CD25+ cells that are T_{regs} (i.e. CD25^{high}) are difficult to detect due to problems differentiating CD25^{high} from CD25^{intermediate}. The transcription factor Forkhead Box P3 (FoxP3) can be used as an additional marker to identify these cells – they are defined as CD4+/CD25+/FoxP3+. There is no evidence of reduced numbers of T_{regs} in MS. Evidence for reduced levels of FoxP3 in MS

exists, together with functional experiments showing impaired T_{reg} function in MS (171, 172). Even if differences have not been found in the absolute number of T_{regs}, the lower level of FoxP3 correlates with reduced function of these T_{regs} (53, 54).

4.1.4.2. B cell findings

4.1.4.2.1. The role of B cells in MS pathology

The dogma of exclusive T cell pathology in MS has been challenged in recent years. The success of a clinical trial of Rituximab in MS (173), followed by Ocrelizumab (174) and Ofatumab (175), has spearheaded a renewed interest in the role of B cells as pivotal players in disease pathogenesis. Furthermore, the discovery of B cell germinal centre-like structures in the brains of MS sufferers (176, 177) and the presence of CSF OCBs in the overwhelming majority of people with MS provides compelling evidence for the importance of B cells in MS, and provides avenues for future study (178).

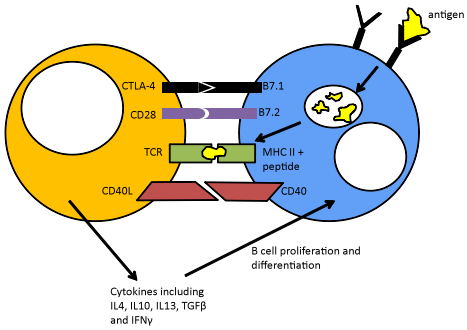


Figure 4.6: T- and B- cell interaction

Most antigens require T-cells for maximal antibody production. Following antigen binding to B-cell receptors, the antigen is engulfed and digested. Antibody fragments are displayed on the B-cell surface bound to the class II MHC complex. T cells which recognise this antigen are then bound via the T cell receptor (TCR). Co-stimulatory interactions then occur. CD40 ligand (CD40L) is expressed on activated T_H-cells, binding to B-cell surface CD40. Together these drive B-cell activation, proliferation and differentiation. Activated B-cells express other co-stimulatory molecules, including surface B7.1 and B7.2 proteins that respectively bind CTLA4 and CD28 on T-cells. Cytokines feedback to direct further activation and maturation steps. IL-4 preferentially induces switching of immunoglobulin production IgE, whereas TGF-β induces switching to IgA. IFN-γ induces IgG production by activated B-lymphocytes. IL-21 is thought to promote the formation of plasma cells.

The role of B cells is not limited to antibody secretion, as they also play a major role in the mediation of T cell responses, through their role in antigen presentation, cytokine production and germinal centre formation (179) (figure 4.6). It has been recognised for many years that B cells are present in the inflammatory plaques that characterise MS (180), but until recently they were not thought to play a major role in plaque formation and propagation. However this viewpoint has shifted, with the discovery of B cell follicle-like structures in the brains of people with late stage secondary progressive MS (181, 182). These structures exhibited some features of germinal centres; however, whether they represent a true germinal centre reaction remains unclear.

It has been postulated that the establishment of these areas with proliferating B cells, plasma cells, follicular dendritic cells and expression of activation-induced cytidine deaminase localised to the brains of patients with MS provides a microenvironment in which B cell maturation, expansion, affinity maturation and immunoglobulin production can occur, thus driving the MS disease process (183). Later work has correlated the presence of these B cell follicle-like structures with severe cortical pathology and an aggressive clinical course, further reinforcing their role in disease progression (177). Grey matter damage in the brains of people with MS consists of both demyelination and axonal degeneration (184). Although the underlying cause of this grey matter atrophy remains unclear, a gradient of damage extending away from meningeal B cell follicle-like structures has been demonstrated (177), possibly implicating these structures in disease progression.

4.1.4.2.2. Markers of CNS B cell activation

Oligoclonal bands (OCBs) detected by isoelectric focussing of serum and CSF are present in around 90% people with MS (185). They are not, however, unique to MS, as they are also present in CNS infections and other CNS localised autoimmune diseases such as paraneoplastic CNS syndromes (186). These bands, which represent IgG unique to the CSF, are a marker of intrathecal B- and plasma cell activity. It has been shown that clonally expanded plasma cells, which can be found in MS lesions and in the CSF of people with MS, are likely to be at least one of the sources of the clonal IgG forming OCBs, although it is unclear whether other clonal populations contribute (187). In MS, the presence of oligoclonal bands in the CSF has been shown to be concomitant with the presence of both B-cells and antibody secreting plasma cells in the CSF (188, 189). Despite extensive searching, no clear single antigen target for these B- and plasma cells has been found (190).

A potential marker of compartmentalised B cell activation in the CNS is the presence of free light chains, which can be found in people with MS and clinically isolated syndromes (41, 43, 191, 192), as discussed previously in section 3.1.1.

4.1.5. Interaction between vitamin D and the immune system

There has been much recent interest in the interaction between vitamin D and the immune system in recent years. This provides a link between the apparent MS risk caused by low vitamin D levels, and the immune abnormalities seen in MS. Vitamin D appears to be able to modulate T-cell differentiation and response in humans. 1α-hydroxylase mRNA is expressed in T-cells, allowing local synthesis of 1,25-dihydroxyvitamin D (193).

Vitamin D appears to be able to alter the differentiation path of T_H0 cells, promoting a T_H2 phenotype at the expense of T_H1 cells. This has been seen in people with RRMS, where vitamin D status correlates with a more T_H2 directed balance (194). The absolute number of T_H17 cells has been correlated with serum 1,25-hydroxyvitamin D levels in MS patients (194). In addition to its effects on T_H cell development, vitamin D is also able to directly attenuate cytokine production by T_H1 and T_H17 cells, further modulating the immune response (195). Vitamin D acts as a suppressor of T_H1 and T_H17 cells (196, 197), and also appears to promote T_{regs}, correlating with suppressive (regulatory) function (198). However, whether this is a direct effect of vitamin D on the T_{reg} population (198), or an indirect effect through the generation of tolerogenic dendritic cells (199) remains unclear. Interestingly, high dose vitamin D supplementation appears to have an effect on the T-cell populations of people with MS (200).

Vitamin D is able to indirectly inhibit B-cell function through its effects on T-cells, and resulting reduction in the T-cell:B-cell interaction and co-stimulation (201, 202). However, it appears to have a direct effect on B-cell biology as well. 1,25-dihydroxyvitamin D has been shown to inhibit B-cell proliferation *in vitro* (203), as well as inducing apoptosis of activated B-cells (203). It is able to induce the expression of CD38, but inhibits B-cell differentiation into plasma cells, which are CD38+/CD27+, and also decreases the production of IgG (203).

4.1.6. A potential causal pathway and endophenotype?

The initial MS risk of an individual can therefore be thought of as the result of a number of factors which are defined at or around the time of birth. These include family history, genetics, sex, and month and place of birth. Initial risk is also influenced by static protective factors, including protective HLA haplotypes. During childhood and adolescence additional dynamic risk factors come into play, altering MS risk with each exposure. These dynamic risk factors include vitamin D deficiency, smoking status and EBV exposure. It is likely that there are also dynamic protective factors acting over the same period, but to date none have been described. It is possible that there is a critical age of action for at least some of these dynamic factors, as demonstrated by the influence of age of migration and the changing risk of MS with age.

However, the order in which these factors act to increase risk is uncertain. They certainly interact with static risk factors. As an individual acquires dynamic risk factors over time, the probability of developing MS increases, until a biological threshold is reached, and demyelinating pathology becomes inevitable. At this point MRI changes in keeping with demyelination appear (the RIS). Favourable and unfavourable disease modifying factors (including the dynamic factors active at an earlier stage) then act and interact within an individual driving disease progression through CIS to clinically definite MS (figure 4.7).

Little has been done to study many of the risk factors described above in a presymptomatic MS population, as this population has traditionally been impossible to identify. Large scale, population based studies have been performed (147, 204), however, these have not focussed on an “at risk” population. This group, which can be defined within the MS endophenotype, includes siblings of people with MS.

Cerebrospinal fluid oligoclonal IgG bands are present in 19% siblings of people with MS but only 4% healthy controls (p=0.02) (92). Asymptomatic MRI abnormalities suggestive of MS are seen in approximately 10% siblings of people with MS (93, 205) compared to 0.06% of the general population (206). Preliminary evidence suggests MS risk in those with MRI evidence of demyelination but without any clinical correlate may be as high as 30-45% at 60 months (207, 208). This indicates that siblings of people with MS do indeed have some endophenotypic markers, and are therefore a valid population to study in order to examine the MS endophenotype.

If we can act to modify some of the dynamic risk factors at an early enough stage – i.e. before point (2) in figure 4.7, when biological disease becomes inevitable, then we may be able to prevent MS. The problem remains as to how to identify individuals at this point in the disease process whilst they are still asymptomatic. Further work is needed to define the order and timepoints at which dynamic risk factors act, and any interactions that may take place between them, in order to inform potential preventative studies in the future.

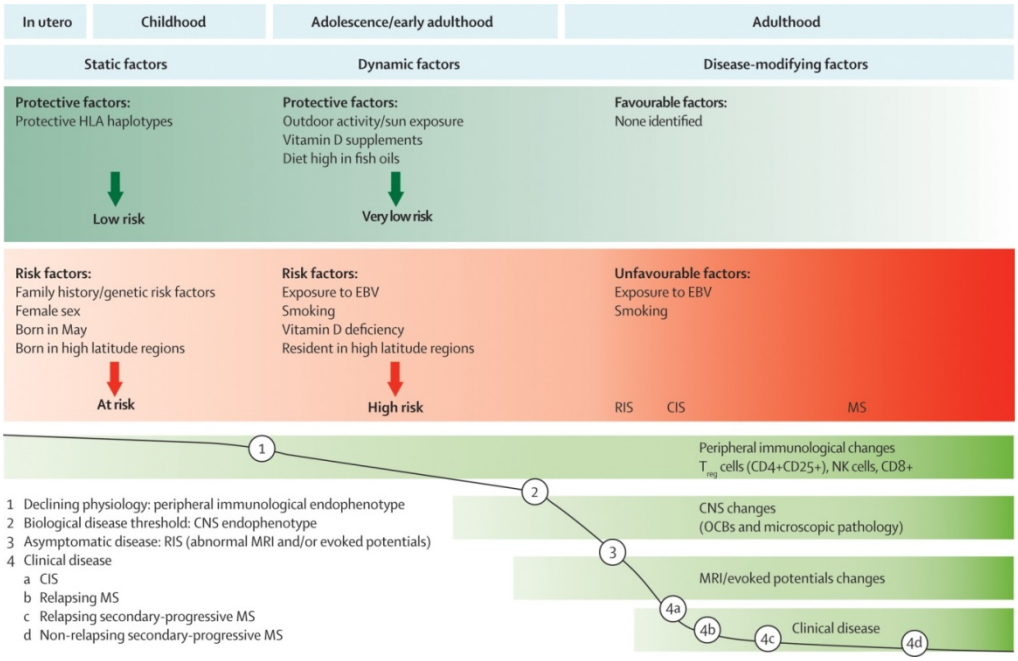


Figure 4.7: Events leading up to the development of MS. Figure taken from Ramagopalan 2010 (2)

4.2. Methods 1: recruitment and sample analysis

4.2.1. Sample size calculation

As this is an exploratory study, power calculations were difficult to perform. The aim was to power the study to detect an endophenotypic fingerprint in the unaffected siblings of people with MS. Previous studies have estimated that 19% siblings of people with MS have OCBs detectable in their CSF (209). Although in this study the siblings of people with MS are not having a lumbar puncture in order to examine for CSF OCBs, it would seem a reasonable assumption that the presence of CSF OCB in this group would be associated with other features suggestive of an endophenotypic trait.

Using the estimated prevalence of 19% (95%CI 8-30%) for CSF OCBs in siblings (the most accurate estimate available from the literature) (209), to get 20 siblings with evidence of a positive endophenotypic fingerprint, 100 siblings would be required. The prevalence of CSF OCBs has been reported as 4% in healthy controls matched to unaffected siblings of people with MS (209). Using these figures, 76 siblings are required to give a power of 0.8 and an alpha of 0.05 to detect a difference in the prevalence of the endophenotypic fingerprint (210). If the prevalence of the endophenotypic fingerprint in siblings is reduced to 15% (again, 4% in healthy controls) then 111 siblings are required to give a power of 0.8 and an alpha of 0.05 using standard power calculations (210). Drawing on data published in the psychiatric literature, most exploratory endophenotypic studies enroll 25-100 participants with an equal number of siblings and controls (211).

Given that the study design requires only a single visit for the majority of participants, there was no need to factor drop-out rates into the power calculation. Only a minority of participants would be invited for a second visit to undergo MRI. All participants were consented to be contacted regarding this second visit this at the start of the study.

4.2.2. Ethical approval

This study had ethical permission granted by the East London REC 1 (ref. 10/H0704/62). An amendment was granted in May 2011 allowing me to recruit the children of people with MS in addition to siblings, although this proved unnecessary, as there were sufficient siblings willing to participate in the study.

4.2.3. Recruitment

All participants were recruited according to ICH-GCP guidelines, and within to the ethical permission that was granted (see section 4.2.2). Full informed consent was taken from all participants.

Participants were recruited from a variety of sources. People with MS attending the MS outpatient clinic at the Royal London Hospital were approached regarding the study, and given brief written information regarding the study to share with their siblings, together with the contact information for myself. If both the person with MS and their siblings were interested in participating they were then sent the participant information sheet (see supplementary appendices 3a-c) and invited to book a visit. Additionally, information regarding the study was provided to patients and families attending the annual Barts MS Research Day. The study was advertised via the MS Research Blog (www.multiple-sclerosis-research.org).

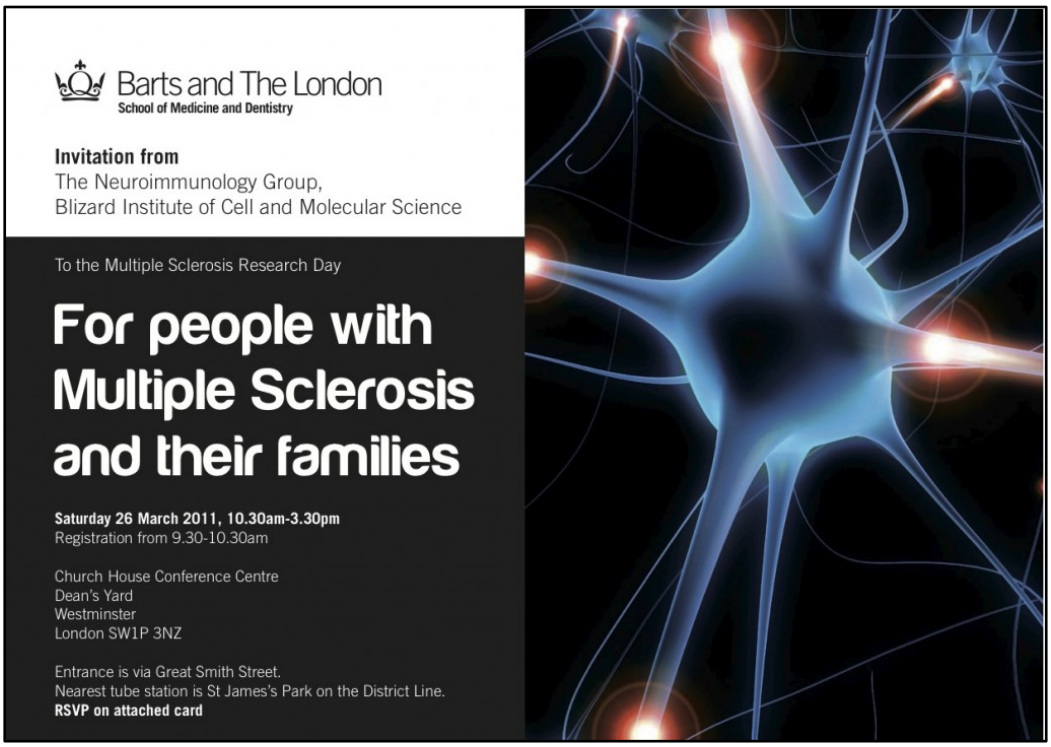


Figure 4.8: Flyer for MS Research day

Various charities also participated in the recruitment drive for this study. The MS Society initially advertised for participants via their website, and later in their print publication; Research Matters. The Multiple Sclerosis Resource Centre also advertised for participants via both their website and their print publication, Pathways.

Recruitment commenced in December 2010 and was completed in January 2012.

4.2.4. Patient visits

All patient visits were performed at the Royal London Hospital, Whitechapel. Siblings were invited to attend either together or separately. All visits followed a standardised protocol; and all visits were performed with myself. I was solely responsible for data and sample collection. Prior to any data or sample collection, full informed consent was taken from all participants. All participants had received the patient information sheet prior to the visit (in most cases at the time of enquiring about the study). Healthy control samples were mainly collected at the Royal London Hospital; additionally a number of healthy controls were recruited via Landor Associates at the workplace of the participants.

Clinical and personal information was gathered via the use of a standardised questionnaire administered by myself. The data collection tool is shown in supplementary appendix 3. All personal data was pseudoanonymised and stored in an encrypted form. All patients and siblings were examined neurologically, and the Expanded Disability Status Scale (EDSS) of participants with MS was calculated. All biological samples were taken using universal precautions and pseudoanonymised at the time of collection. Urine samples were tested for the presence of nitrites at the time of sampling using widely available urine dipsticks, and those samples with evidence of asymptomatic bacterial infection discarded. All samples were collected on the day of the patient visit, and all samples were processed on the same day that they were taken.

4.2.5. Laboratory methods: sample processing

4.2.5.1. Serum and plasma separation

Peripheral blood was collected into 1x BD red top serum tube (catalogue no. 367837) and 3x BD green top lithium/plasma tubes (catalogue no. 368480). Samples were stored at room temperature until processing, which was performed on the same day as sample collection, usually within 1-3 hours.

The serum (red top) tube was centrifuged at 1000g for 10 minutes. The serum was then aspirated from the tube and stored in 3 aliquots at -80^{o}c until batch analysis. Blood from the lithium/plasma tube was transferred to a single sterile 50ml falcon tube, and centrifuged at 500g for 10 minutes. 3ml of plasma was then aspirated, taking care not to disturb the white-cell rich buffy coat, and re-centrifuged at 1000g for 10 minutes in order to remove debris. The plasma was then separated into 3 aliquots and stored at -80^{o}c until analysis. The remaining blood sample was then processed to separate the mononuclear cells (see section 4.2.5.2).

4.2.5.2. Peripheral blood mononuclear cell (PBMC) separation

Peripheral blood mononuclear cells were separated using a standard technique over a lymphoprep (Ficoll) gradient. Peripheral blood was collected into 3x BD green top lithium/plasma tubes (catalogue no. 368480). 3 x 1.5ml cryovials of whole blood were removed prior to any further processing and stored at -80^{o}c. The remaining blood was transferred into a sterile 50ml Falcon tube and plasma collected (see section 3.2.5.1 for details). Following the removal of 3ml plasma, the remaining blood was diluted to a final volume of 50ml using PBS warmed to room temperature. 15ml lymphoprep (warmed to room temperature) was placed in a 50ml Falcon tube, and 25ml diluted blood carefully layered on top of the lymphoprep. This was then centrifuged at 800g with the brakes off the centrifuge for 30 minutes at room temperature.

After centrifugation, mononuclear cells and platelets could be seen to form a visible whitish layer just under the plasma layer. This cell layer was harvested using a Pasteur pipette. The cells were transferred to a sterile 50ml Falcon tube and made up to 50ml using PBS. These cells were then washed twice prior to freezing.

The separated cells were divided into three aliquots prior to freezing and storage. Cells were suspended in freezing media (FCS+ 10%DMSO) and divided into 3 aliquots. They were frozen slowly at a rate of -1^{o}c/hr using a CoolCell container, and transferred to the liquid nitrogen cryostore in batches. The time between freezing to -80^{o}c and transfer to the liquid nitrogen cryostore was not longer than 1 month in all cases.

4.2.5.3. Urine sample processing

Mid-stream urine samples were collected, reducing the risk of bacterial contamination of samples. As detailed above, samples were tested for the presence of nitrites at the time of sampling, and discarded if evidence of bacterial infection of the urinary tract was present. Samples were divided into three aliquots prior to freezing at -80^{o}c. Care was taken to ensure that samples were not exposed to direct light for prolonged periods of time, in order to allow neopterin levels to be accurately assessed at a later date.

4.2.6. Selecting the most accurate ELISA for assessing anti-EBNA-1 IgG status

4.2.6.1. Why was this preliminary work required?

A number of techniques have been developed for detecting and quantifying anti-EBNA-1 IgG, of which the gold standard is indirect immunofluorescence (212). However, this technique is not readily amenable to large scale testing, and has fallen out of widespread use. The DiaSorin EBNA-1 IgG ELISA (DiaSorin; Salugia, Italy) has reasonable sensitivity (87.5%) and specificity (89.9%), and correlates well with the results obtained by indirect immunofluorescence (212). Indeed, the DiaSorin platform is used by the clinical microbiology laboratory at the Royal London Hospital (personal communication, Dr Duncan Clark).

There are no international consensus units for the measurement of anti-EBNA-1 IgG titres, instead manufacturers define arbitrary units, making comparisons complex. Rank quintiles are an accepted method of comparing titres between groups when examining continuous variables and this technique has been used in MS epidemiology (133, 147).

Despite the fact that sensitivity and specificity of some of the ELISA kits available to detect EBNA-1 have previously been demonstrated to be variable (212, 213), no quantitative analysis has been performed, and current assays have not been compared. The assay in current use in the medical microbiology laboratory at the Royal London Hospital, produced by DiaSorin (DiaSorin; Salugia, Italy) was therefore compared to the assay in current use in the neuroimmunology group, produced by VirionSerion (VirionSerion; Wurzburg, Germany). Specificity, sensitivity and both absolute values and rank quintiles were compared between the two assays.

4.2.6.2. Assay protocols

120 serum samples (38 patients with MS, 50 siblings of patients with MS and 32 healthy controls, HC) were used in this preliminary study. All samples were assayed in duplicate.

DiaSorin EBNA-1 IgG ELISA was performed according to the manufacturer's instructions. Prior to use of the VirionSerion EBNA-1 IgG ELISA, samples were diluted 1:5 using the dilution buffer provided by the manufacturer in order to obtain readings within range; otherwise the assay was performed according to the manufacturer's instructions.

Anti-EBNA-1 IgG titres for each sample were calculated according to the manufacturers' instructions. Samples were classified as positive or negative using the cut-off values provided. Samples with titres higher than the upper limit of detection for each kit were assigned a value equal to the upper limit of detection. Repeat analysis on borderline positive/negative samples was performed and the average measurement was used to determine sample positivity. Positive samples were divided into quintiles using rank order in order to assess the rank agreement between ELISA kits.

4.2.6.3. Statistical methods to describe agreement between assays

In the sensitivity and specificity analysis the results obtained using the DiaSorin ELISA were designated as the gold standard for the reasons discussed above. In all other analyses the results from the two ELISA kits were directly compared. Analyses were performed using Prism v5 (GraphPad).

The kappa coefficient was used to describe the correlation between the rank quintiles generated by the two ELISAs. The agreement between the two methods was assessed using a Bland-Altman plot (214). This method allows a visual description of both the agreement between ELISAs, in addition to demonstrating any systematic or significant proportional errors between the two sets of results (214). In a Bland-Altman plot, the mean value for each sample is plotted on the x-axis, with the difference between the two values obtained for each sample plotted on the y-axis. The mean difference between the two results (the mean y-axis value) gives the average agreement between the two ELISAs, and the distribution of results gives an indication of any systematic error.

4.2.6.4. Results of the ELISA comparison

The mean coefficient of variation (CV) of positive samples was 3.8% with the DiaSorin ELISA and 12.7% with the VirionSerion ELISA. The positive predictive value of the VirionSerion ELISA was 99.1%, however the negative predictive value was 64.3% when calculated compared to the DiaSorin ELISA. Sensitivity and specificity were 95.5% and 90.0% respectively. Details regarding the number of samples detected as positive and negative, and the number of samples giving rise to readings greater than the upper limit of detection, are given in table 4.1.

When comparing the values obtained with the two different ELISAs, only the samples which gave positive values in both ELISAs were used in the calculations. When the raw values were used, there was a poor correlation between the results obtained from the two ELISAs, R^2=0.49 (figure 4.9a). The relationship between the two kits appeared exponential rather than linear (figure 4.9b). When the positive samples were ranked into quintiles, the kappa coefficient was 0.23 (95% CI 0.11-0.35) (table 4.2). The kappa coefficient improved to 0.47 when linear weighting was used (see table 4.2).

Given that both ELISAs should be consistent with calling samples either positive or negative, the decision was taken not to examine the magnitude of the error in this study. Instead, information regarding the positive (99.1%) and negative (64.3%) predictive values should be used to compare those samples. The implication of these values is that whilst the DiaSorin and Virion ELISA agree on those samples that DiaSorin calls positive, there is a significant overestimation of the proportion of negative samples by the Virion ELISA.

A Bland-Altman plot (figure 4.10) demonstrated poor agreement between the results generated by each ELISA, with a mean difference between ELISAs approaching 50% of the maximum value. There was also significant skew indicating a proportional error between the two kits.

These results have clear relevance for the endophenotype study. The CV for the VirionSerion ELISA (12.7%) is sufficiently high as to call the accuracy of the results into question. Although the sensitivity of the VirionSerion ELISA appears adequate when compared to the DiaSorin ELISA, the low negative predictive value is a major concern, especially given the fact that that DiaSorin ELISA has previously compared favourably to indirect immunofluoresence, the gold standard.

Table 4.1: Titres, number of positive/negative samples and number of samples with values greater than the upper limit of detection.

Group (n)	Age (mean; range)	Gender (m:f)	DiaSorin EBNA-1 IgG titre[1] (mean IU; SD)	Virion EBNA-1 IgG titre[1] (mean IU; SD)	Positive DiaSorin EBNA-1 IgG (n; %)	Positive Virion EBNA-1 IgG (n; %)	DiaSorin EBNA-1 >ULD[2] (n; %)	Virion EBNA-1 >ULD[2] (n; %)
MS (38)	43.9 (20-65)	3:35	165.0; 57.6[3]	3290.3; 3208.6[4]	36; 95%[5]	37; 97%[6]	9; 25%[7]	5; 13%[7]
Siblings (50)	43.0 (18-67)	15:35	140.9; 66.2	2480.3; 2989.1	44; 88%	41; 82%	7; 16%	4; 8%
HC (32)	38.6 (24-55)	9:23	140.2; 57.3	2043.9; 2667.6	30; 94%	27; 84%	3; 10%	2; 6%

1: Mean of all positive samples, negative samples excluded
2: Upper limit of detection; positive samples only included (negative samples excluded)
3. p=0.05 for difference between MS and pooled HC and siblings, no significant difference between MS and HC (unpaired t-test)
4: No significant difference between MS and either HC or pooled HC and siblings (unpaired t-test)
5: No significant difference between MS and either HC or pooled HC and siblings (Fisher's exact test)
6: p=0.035 for difference between MS and pooled HC and siblings, no significant difference between MS and HC (Fisher's exact test)
7: No significant difference between MS and either HC or pooled HC and siblings (Fisher's exact test)

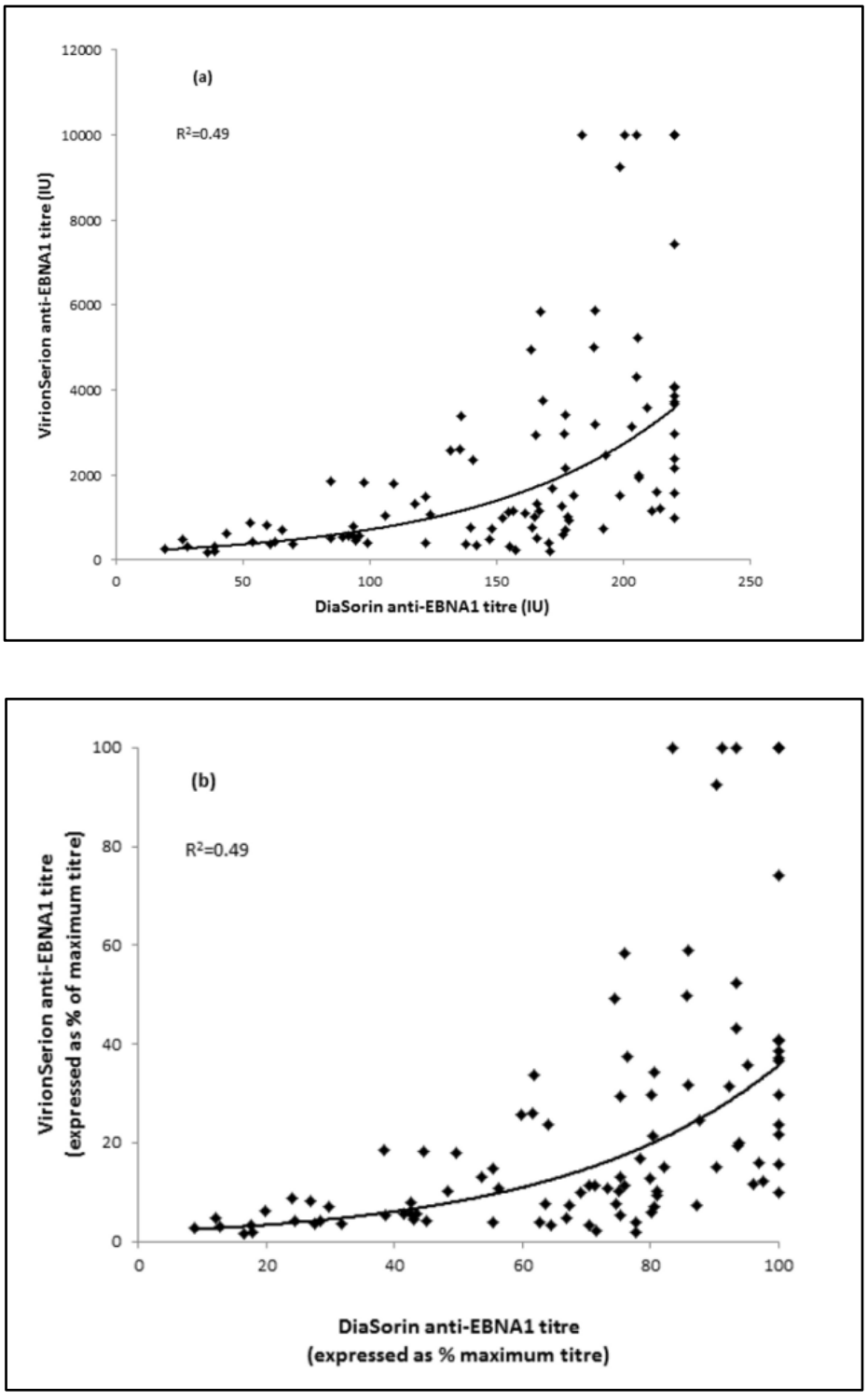


Figure 4.9: Scatter graph demonstrating the poor correlation between the DiaSorin and VirionSerion ELISA results. Only those samples giving positive results with both ELISAs are shown. **(a)** Correlation shown according to absolute results. **(b)** Results standardised to percentage of maximum value in order to allow more direct comparison between ELISAs. These graphs include the samples that were recorded as seropositive by both ELISAs only. The positive and negative predictive values are used to compare the differences in the proportion of samples where there was a discrepancy regarding seropositivity.

Table 4.2: Agreement between results from the two ELISAs when the positive samples are arranged into rank quintiles.

		VirionSerion Rank Quintile				
DiaSorin Rank Quintile		**1**	**2**	**3**	**4**	**5**
	1	10	5	0	0	1
	2	6	7	6	4	0
	3	6	3	7	4	2
	4	0	4	4	6	8
	5	0	1	3	7	10

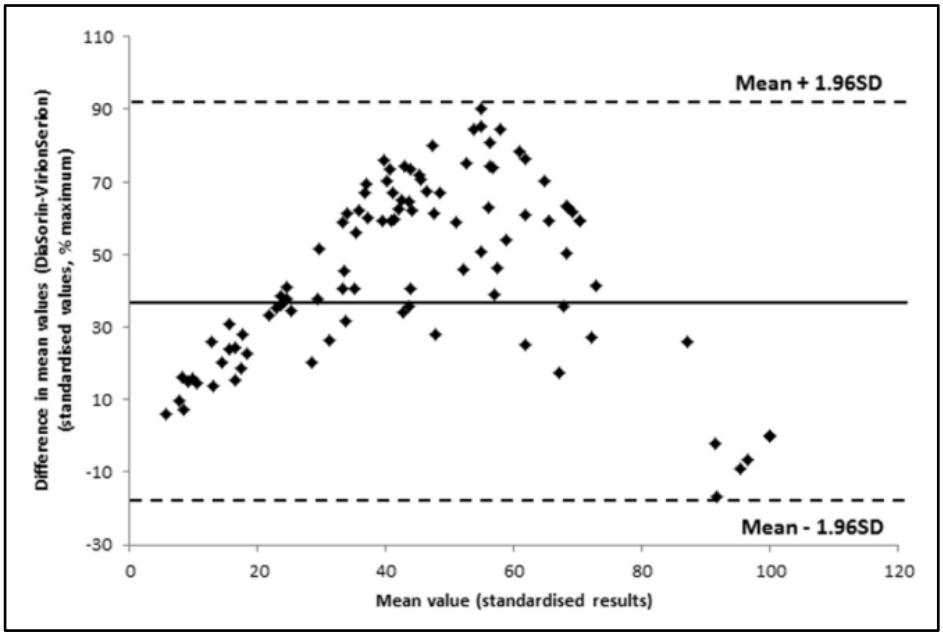


Figure 4.10: Bland-Altman plot demonstrating the poor agreement and proportional error between the DiaSorin and VirionSerion ELISA results. Only those samples giving positive results with both ELISAs are shown. Mean values are taken from the standardised results (percentage of maximum value) for both ELISAs.

Both ELISA kits used in this study employ the EBV p72 antigen; however the method of antigen production differs. DiaSorin use a synthetically manufactured version of the antigen, whilst VirionSerion employ a recombinant antigen. It is unclear as to whether this affects overall assay performance, but should be borne in mind when assessing assays.

The decision was therefore taken to employ the DiaSorin ELISA, rather than the cheaper VirionSerion kit which had previously been in use in the neuroimmunology laboratory, for the analysis of all samples in this study.

4.2.7. Anti-EBNA-1 IgG quantification and use of quintiles

As discussed above, the commercially available ELISA produced by DiaSorin was used to determine anti-EBNA-1 IgG titres. Samples were analysed in batches on 96-well plates according to the manufacturers' protocol. Samples were thawed on the day of analysis, and had not previously been subject to repeated free-thaw cycles. Standards were analysed on each plate, and the values used to determine the IgG titre for each serum sample. All samples were assayed in duplicate. Quality control samples were also run on each plate. Borderline samples (i.e. those giving values within 10% of the positive/negative cutoff value), and samples with a coefficient of variation >10% were repeated. In the case of borderline samples, if both runs gave a positive IgG titre then the sample was called as positive, with a value equal to the mean of all four analyses. There were no samples where one run gave a positive result and the other gave a negative result.

Once all samples had been analysed, the pooled positive IgG titres from the sibling and healthy control groups were used to define quintiles. As stated above, the units used to describe titres are manufacturer specific, and so in order to use relative risks associated with non-MS quintiles of IgG titres, these must be defined for each study population and analysis platform used.

4.2.8. Defining smoking status

Two methods were used to define smoking status. Participants were directly questioned as to whether they were current or ex-smokers, and if so, how heavily they smoked. However, there is evidence that replying on self-reported smoking behavior may lead to an underestimation of the true prevalence of smoking (215). Biochemical validation of smoking status is commonly used to improve the accuracy of self-reported status in studies of smoking exposure and smoking-related health risks and in studies of cessation interventions (216).

Cotinine is the major metabolite of nicotine. It has been widely used as a biomarker of exposure to tobacco, and therefore to define those with exposure levels of active smokers (217). However, there is an overlap in cotinine values between less intense or less frequent active smokers, and people heavily exposed to secondhand smoke (i.e. those living with heavy smokers). The most widely used cutoff point for discriminating smokers from non-smokers is 14ng/ml (218). However, given recent smoke-free legislation, indoor exposure to areas with high concentrations of tobacco smoke has markedly declined in the UK, and so a lower cutoff is likely to be more accurate. A recent large-scale analysis of 3,078 smokers and 13,078 nonsmokers demonstrated that a cutoff of 3.08ng/ml gave the highest sensitivity and specificity for distinguishing smokers from non-smokers (216). This value was therefore used in the analysis.

Serum cotinine was measured using a commercially available ELISA (Calbiotech; California, USA). The ELISA was performed according to the manufacturers' instructions on samples that had not been previously thawed. As detailed above, a value of 3.08ng/ml was used as the cutoff value; subjects with a serum level greater than this were defined as smokers. Those with a level below this who reported a previous smoking history were defined as ex-smokers or ever-smokers, those with a level <3.08ng/ml who reported never smoking were defined as never smokers. Those participants with a cotinine level ±2ng/ml of the cut-off point had their serum samples re-assayed in duplicate, and the

mean of all of the results for that individual participant were taken into account when defining them as either a biochemical smoker or non-smoker.

4.2.9. Vitamin D

4.2.9.1. Measuring serum 25-hydroxyvitamin D levels

Serum 25-hydroxyvitamin D levels were measured in collaboration with the Department of Clinical Biochemistry, Royal London Hospital, Whitechapel. 25-hydroxyvitamin D (25-OHvD) has a half-life of approximately 3 weeks; it forms the predominant circulating form of vitamin D in the normal population. Levels of 25-OHvD are considered to be the most reliable indicator of overall vitamin D status in an individual.

25-OHvD was measured using liquid chromatography-tandem mass spectrometry on serum samples that had been frozen on the day of sampling and had not been previously thawed or exposed to direct light for extended periods of time. Measurements were made using a fully automated system, the TECAN EVO 100, on a Walters Acquity-Quattro Premiere XE LCMS-MS system. Samples were analysed in batches using 96-well plates, with standards and quality control samples run alongside each batch. Serum levels of 25-OHvD were then calculated using the standards run in the same batch as the samples in being analysed.

4.2.9.2. Correcting for seasonal variation in serum 25-hydroxyvitamin D levels

Samples collected as part of the sibling study were collected throughout the year. An estimation of the potential effect of seasonal variation on serum 25-OHvD levels was made by collecting healthy control serum samples at the end of winter (April 2010) and end of summer (September 2010). There was a highly significant difference between serum 25-OHvD levels between these dates in an overlapping cohort of individuals (p=1.35x10^-8; t-test on log values) (figure 4.11). At the end of winter, 77.8% of subjects did not have sufficient vitamin D levels, whereas this proportion fell to 12.0% at the end of summer (p<0.0001; Fisher's exact test; raw numbers used in calculation).

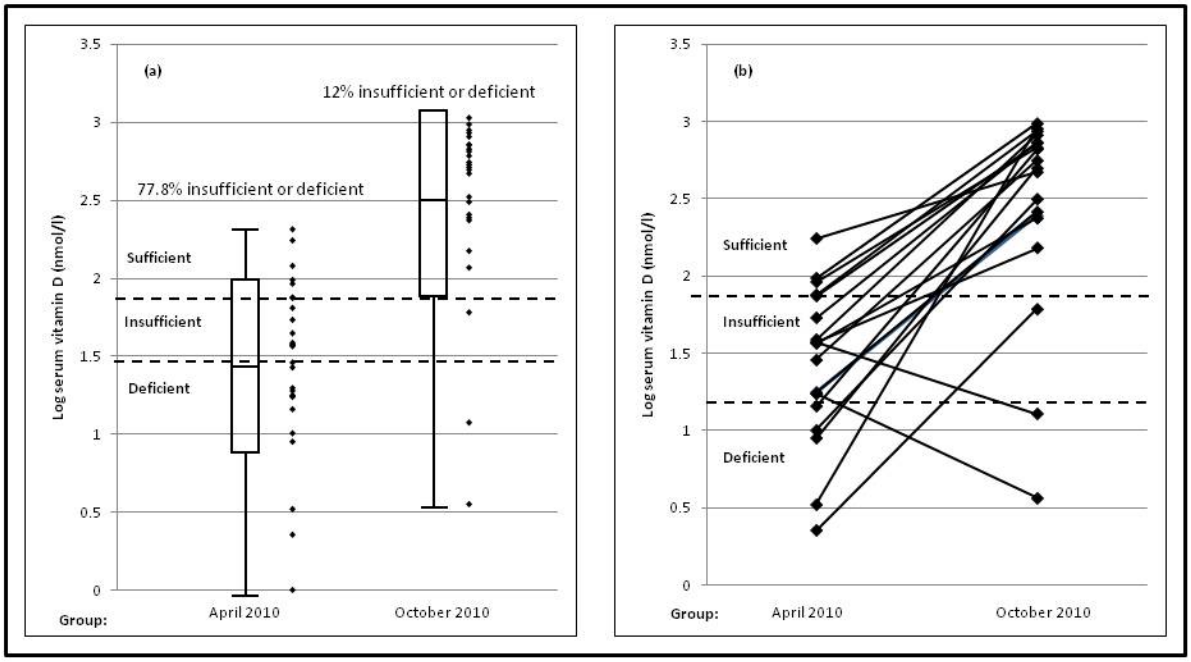


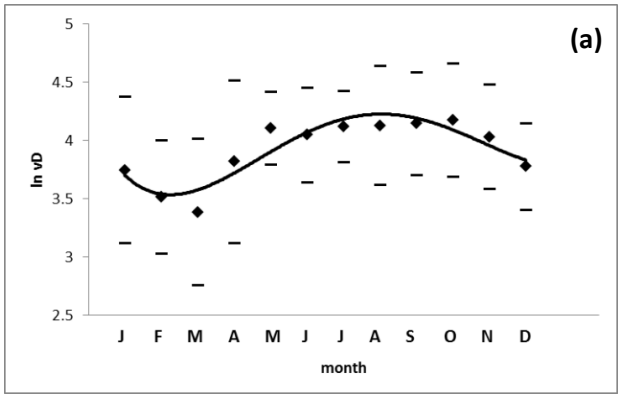
Figure 4.11: (a) Combined scatter and box-and-whisker plot showing log serum 25-hydroxyvitamin D levels (nmol/L) at the end of winter and end of summer. The box indicates the mean +/- 1 standard deviation, and the whiskers the range. The dashed lines indicate the accepted levels for deficiency (<25nmol/l), insufficiency (25-75nmol/l) and sufficiency (>75nmol/l). **(b)** Graph showing the change in serum 25-hydroxyvitamin D levels (nmol/l) between the end of winter and end of summer between paired samples (i.e. the same subjects, rather than matched pairs, therefore not all subjects shown).

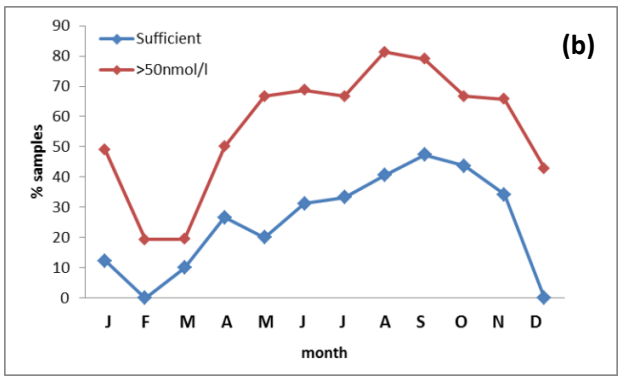
In order to validate the need to correct for seasonal fluctuations in 25-OHvD, the MS, sibling, and healthy control samples forming the study cohort were plotted against the month in which the

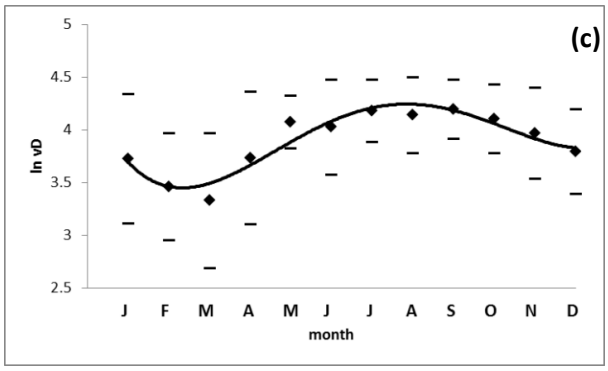
samples were taken (figures 4.12a and 4.12b; table 4.3). Whilst the expected sinusoidal dip following the winter months can still clearly be seen, the expected peak following the summer months is not seen. In order to determine whether an MS-specific effect could be responsible for this lack of 25-OHvD peak, the sibling and HC samples only were analysed. Figure 4.12c, where the variation in 25-OHvD levels in the non-MS population are plotted over the year, and figure 4.12d, where the variation in the non-MS population is superimposed on the results from the entire study cohort demonstrate that this is clearly not an MS-specific effect, rather an entire-population effect.

Table 4.3: Serum 25-OHvD levels stratified by month of collection

Month	Mean 25-OHvD (SD) (nmol/l)	% samples >75nmol/l	% samples >50nmol/l
January	50.22 (27.93)	12.24	48.98
February	37.22 (16.28)	0	19.35
March	35.95 (24.78)	10.00	19.51
April	55.87 (33.77)	26.67	50.00
May	63.40 (21.59)	20	66.67
June	61.38 (21.13)	31.25	68.75
July	63.83 (19.44)	33.33	66.67
August	68.19 (26.97)	40.63	81.25
September	67.79 (23.02)	47.37	78.95
October	71.50 (30.52)	43.75	66.67
November	61.47 (25.08)	34.21	65.79
December	46.14 (17.18)	0	42.86







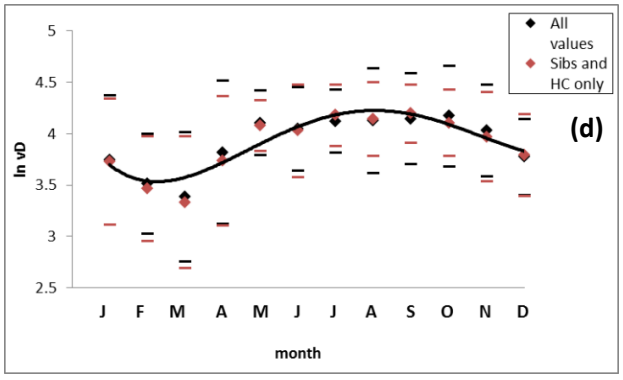


Figure 4.12: (a) Mean ± standard deviation serum 25-OHvD levels, pooled analysis. The sinusoidal dip following the winter months can be seen, however the expected summer peak is not present.

(b) The seasonal variation in vitamin D levels is also clearly seen when looking at the percentage of samples taken each month that are either sufficient (>75nmol/l) or not deficient (>50nmol/l).

(c) Mean ± standard deviation serum 25-OHvD levels, unaffected sibling and healthy control samples only.

(d) Mean ± standard deviation serum 25-OHvD levels, with entire population results shown in black and unaffected sibling and healthy control results overlaid in red. It can clearly be seen that the lack of the summer peak in serum 25-OHvD levels is not caused by the inclusion of the MS population, instead this appears to be an entire-population effect.

An initial regression by month of sampling was performed using the technique described by Munger et al (147). Briefly, the serum 25-OHvD level for each individual was regressed on the periodic function *"—sine (2πX/12) – cosine (2πX/12)"*, where X is the month in which the sample was taken. The residual from the regression added to the original value to give a deseasonalised value. When the values obtained by this model were plotted against month of blood draw however, there was still significant seasonal variation apparent (figure 4.13).

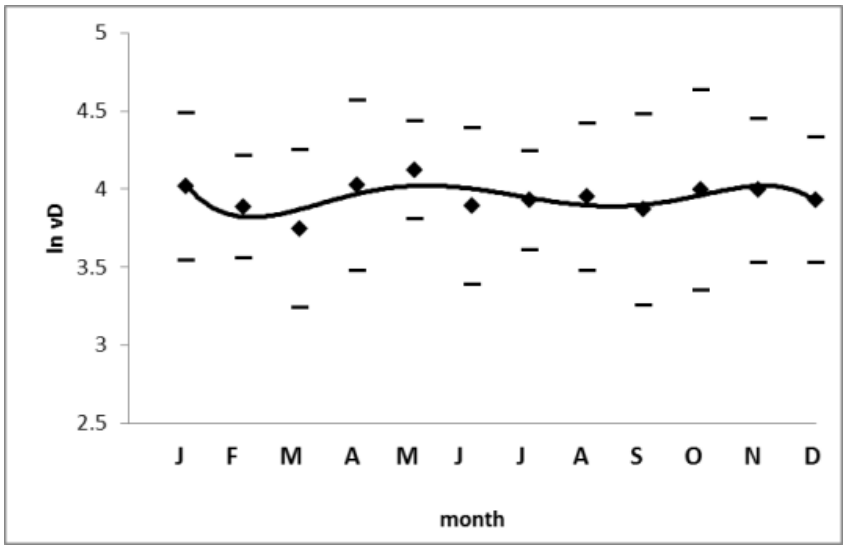


Figure 4.13: Mean ± standard deviation serum 25-OHvD levels deseasonalised by month of blood draw. There is still some seasonal variation apparent in serum 25-OHvD levels.

A regression by day of sampling on the original serum 25-OHvD levels was therefore performed (219). The serum 25-OHvD level for each individual was regressed on the periodic function *"—sine(X/365)*2π – cosine (X/365)*2π "*, where X was the day of the year on which the sample was taken. The residual from this regression was added to the original serum 25-OHvD levels to give the deseasonalised value. When the deseasonalised serum 25-OHvD values were plotted against the day of the year on which they were taken the seasonal variation (figure 4.14a) was no longer apparent (figure 4.14b).

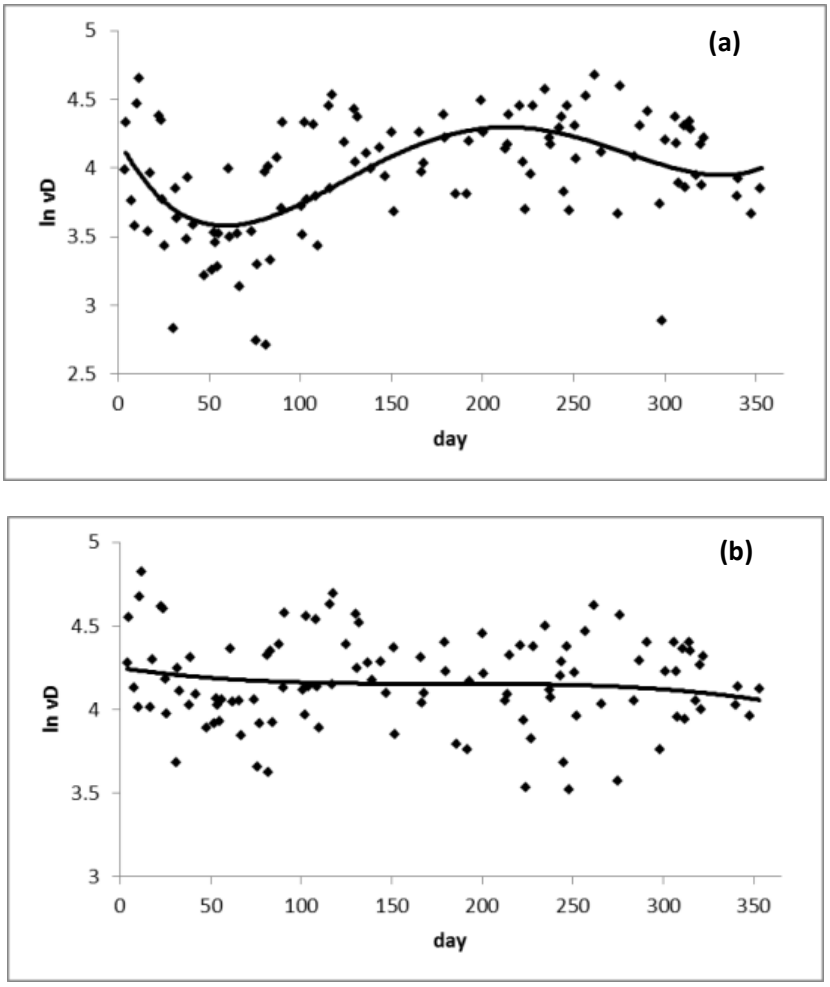


Figure 4.14: (a) Variation in 25-OHvD values by day of blood sampling. **(b)** Following the correction for the day of sampling detailed in the text, the sinusoidal variation in the serum 25-OHvD levels over the course of the year is no longer apparent.

The 25-OHvD values obtained following correction for day of blood sampling were therefore used in the final analysis.

4.2.9.3. Use of quintiles of serum 25-OHvD levels

Once the deseasonalised serum 25-OHvD levels had been determined, they were related to the quintiles defined by Munger et al (147). Although this meant that few of the subjects in this study were placed in the highest quintile, the relative risks given in this seminal publication relate to the quintiles defined by the authors. The highest quintile of 25-OHvD described by Munger et al is higher than would be expected in the UK population, probably due to the nature of the population studied – a large proportion of US military bases are in the southern states, and it could be argued that soldiers are more likely to spend time outside than the general population.

4.2.9.4. Determining whether any interaction exists between treatment with interferon-beta and serum 25-hydroxyvitamin D levels

Some authors have suggested that interferon-β used to treat MS acts to increase 25-OHvD production by the skin in response to sunlight (220). If this was the case in this study cohort, it would have the potential to skew the 25-OHvD levels in the MS population, in turn affecting the overall risk score calculation. In order to assess whether interferon-β usage significantly affected serum 25-OHvD levels in my population, deseasonalised 25-OHvD levels were compared between those people with MS who were taking interferon-β and those who were either taking other therapies or untreated.

As can be seen in figure 4.15, there is no significant difference in deseasonalised 25-OHvD levels between those on interferon-β and those on other therapies or untreated (p=0.118; t-test on log values). There was therefore no need to perform an adjustment for treatment on the deseasonalised serum 25-OHvD levels.

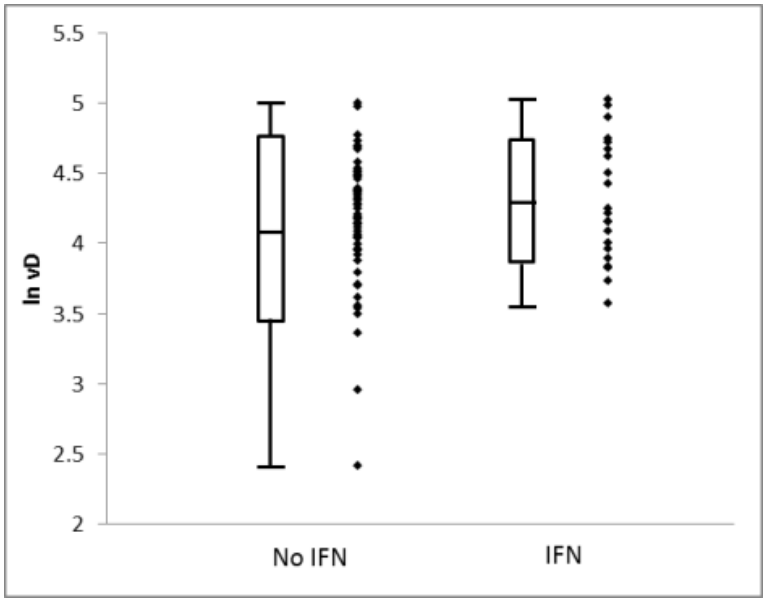


Figure 4.15: Comparison of serum 25-OHvD levels between those on interferon-β treatment and those on either other disease modifying therapies or on no treatment. The box indicates the mean +/- 1 standard deviation, and the whiskers the range. There is no significant difference between the two groups.

4.2.10. DNA isolation and genotyping

4.2.10.1. DNA isolation

DNA isolation was performed from whole blood by the Genome Centre, Queen Mary University London using the BRIGHT protocol based on the salting out procedure published by Miller et al (221). The standard operating procedure for 9ml whole blood, which was adjusted for the 1.5ml whole blood available, is given in appendix 5.

4.2.10.2. Determination of MS genetic risk using the Illumina Immunochip

The Illumina immunochip has been developed using information derived from large-scale GWAS in autoimmune diseases. It is an Illumina Infinium genotyping chip, which contains 196,524 polymorphisms (718 small insertions or deletions and 195,806 SNPs) that have been associated with autoimmune disease (222). It was designed to perform both deep replication of major autoimmune and inflammatory diseases, and fine-mapping of established GWAS significant loci (222). Using data generated by the Wellcome Trust Case-Control consortium, it contains up to 3000 SNPs per disease that have been associated with a variety of autoimmune diseases including multiple sclerosis. Of the 53 genes that were associated with MS in the most recent GWAS (103), 51 of them are represented on the immunochip (see table 4.4).

Immunochip typing was performed at the Genome Centre, Queen Mary University London according to the protocols supplied by Illumina. Briefly, following DNA denaturing, the entire genome is amplified overnight prior to fragmentation. The fragmented DNA is precipitated and resuspended before being hybridised to the bead chip supplied by Illumina. The hybridized gene chip is then stained and read, providing the results for all 196,524 polymorphisms.

Using immunobase (http://www.immunobase.org) the Immunochip markers for each SNP were determined. The two SNPs that were not on the immunochip did not have alternative markers in sufficiently strong linkage disequilibrium to permit determination. Details of the markers together with the associated odds ratio determined in the most recent GWAS (103) are given in table 4.4.

SNPs in strong linkage disequilibrium with the four HLA haplotypes associated with an alteration in MS risk were determined from the paper by de Bakker et al (223). The tagging SNPs chosen all had an R^2 of 1.0 with the HLA allele in question. These are detailed in table 4.5.

Table 4.4: SNPs associated with MS available on the Illumina Immunochip.

SNP identified in GWAS	Associated candidate gene	Immunochip marker name (if different)	Risk allele	Odds Ratio associated with risk allele
rs1315388	HLA-DRB1*1501		A	3.1
rs4648356	MMEL1	imm_1_2699024	C	1.14
rs11810217	EV15	1kg_1_92920965	A	1.15
rs11581062	VCAM1		G	1.12
rs1335532	CD58	imm_1_116902480	A	1.22
rs1323292	RGS1	imm_1_190807644	A	1.12
rs7522462	C1orf106(KIF21B)	imm_1_199148218	G	1.11
rs12466022	no gene	1kg_2_43212565	C	1.11
rs7595037	PLEK	imm_2_68500599	A	1.11
rs17174870	MERTK		G	1.11
rs10201872	SP140	imm_2_230814968	A	1.14
rs11129295	EOMES	1kg_3_27763784	A	1.11
rs669607	no gene	1kg_3_28046448	C	1.13
rs2028597	CBLB		G	1.13
rs2293370	TMEM39A/CD80	imm_3_120702624	G	1.13
rs9282641	CD86		G	1.21
rs2243123	IL12A	imm_3_161192345	G	1.08
rs228614	NFKB1		G	1.09
rs6897932	IL7R	imm_5_35910332	G	1.11
rs4613763	PTGER4	imm_5_40428485	G	1.2
rs2546890	IL12B	imm_5_158692478	A	1.11
rs12212193	BACH2	imm_6_91053490	G	1.09
rs802734	THEMIS	imm_6_128320491	A	1.1
rs11154801	MYB/AHI1		A	1.13
rs17066096	IL22RA2		G	1.14
rs13192841	no gene	imm_6_138008907	A	1.1
rs1738074	TAGAP	imm_6_159385965	G	1.13
rs354033	ZNF746		G	1.11
rs1520333	IL7		G	1.1
rs4410871	MYC		G	1.11
rs2019960	PVT1	imm_8_129261453	G	1.12
rs3118470	IL2RA	imm_10_6141719	G	1.12
rs1250550	ZMIZ1	imm_10_80730323	A	1.1
rs7923837	HHEX		G	1.1
rs650258	CD6	imm_11_60588858	G	1.12
rs630923*	CXCR5		C	1.12
rs1800693	TNFRSF1A	imm_12_6310270	G	1.12
rs10466829	CLEC1	imm_12_9767358	A	1.09
rs12368653	CYP27B1	imm_12_56419523	A	1.1
rs949143	ARL6IP4	imm_12_122161116	G	1.08
rs4902647	ZFP36L1	imm_14_68323944	G	1.11

rs2300603	BATF		A	1.11
rs2119704	GALC/GPR65		C	1.22
rs2744148	SOX8		G	1.12
rs7200786	CLEC16A	imm_16_11085302	A	1.15
rs13333054	IRF8	imm_16_84568534	A	1.11
rs9891119	STAT3	imm_17_37761506	C	1.11
rs180515	RPS6KB1		G	1.09
rs7238078	MALT1		A	1.12
rs1077667	TNFRSF14		G	1.16
rs8112449	TYK2/CDC37	imm_19_10381064	G	1.08
rs874628	MPV17L2		A	1.11
rs2303759	DKKL1		C	1.11
rs2425752	CD40		A	1.11
rs2248359	CYP24A1		G	1.12
rs6062314	ZBTB46/TNFRSF6B		A	1.16
rs2283792	MAPK1		C	1.1
rs140522*	SCO2		A	1.1

* not available on immunochip; no markers in strong linkage disequilibrium

Table 4.5: SNPs in strong linkage disequilibrium with HLA haplotypes associated with an alteration in MS risk

HLA haplotype	Associated SNP	Risk allele	Relative risk associated with HLA haplotype
HLA-DRB1*1501	rs1315388	A	3.1
HLA-DRB1*1303	rs2516049	C	2.4
HLA-DRB1*0301	rs3129763	A	1.26
HLA-A*0201	rs4713240	G	0.73

4.2.11. Urinary free light chains

Free light chains were determined on urinary samples using a commercially available ELISA (BioVendor; Brno, Czech Republic). The assay was carried out to the manufacturers' instructions, and all samples were assayed in duplicate. Urinary protein was measured using the SigmaAldrich total protein kit (SigmaAldrich, St Louis MO, USA), using a Coomassie blue (brilliant blue G) reagent, which does not cross-react with creatinine. Urinary FLC levels were expressed as a ration to total protein to correct for both variable glomerular filtration and urine concentration (see section 3.2.3 for further discussion regarding laboratory techniques).

4.2.12. Other ELISA

4.2.12.1. Neopterin

Urinary neopterin levels were measured using a commercially available ELISA (IBL, Hamburg, Germany) according to the manufacturer's instructions, with all samples assayed in duplicate. Samples were not been exposed to light for prolonged periods, and the assay was performed in the dark. Urinary creatinine was measured using a commercially available kit (R+D Systems; Minneapolis, USA), and neopterin was expressed as a ratio to creatinine to correct for variable urine concentrations (see section 3.2.3).

4.2.12.2. Matrix metalloproteinase-9 (MMP-9)

Matrix metalloproteinase-9 (MMP-9) is one of a family of zinc-containing and calcium requiring endopeptidases, which are capable of remodeling and degrading the extracellular matrix (224). Their activity is regulated at post-transcriptional level by the suppressor function of tissue inhibitors of metalloproteinases (TIMPs), which bind to either the activated MMPs or their proforms (224). Elevated serum concentrations of MMP-9 have been found in serum and CSF of people with MS (225, 226), with higher levels in relapsing remitting MS (225). Increased levels have also been associated with MRI evidence of inflammatory disease activity (227, 228). Conversely, serum and CSF levels of TIMP-1 are lower in people with MS than controls (227, 228). It has therefore been proposed that the MMP-9:TIMP-1 ratio may have a role as a surrogate marker of inflammatory disease activity in MS (228, 229).

There has been considerable debate as to whether peripheral MMP-9 concentrations should be measured in serum or plasma. It has previously been shown that MMP-9 levels are more elevated in serum than in plasma, and that they gradually increase in plasma in the first 12 hours after sample collection (230), possibly due to release from blood cells during platelet activation. It has also been shown that the chelating and clotting agents used during blood sampling have a considerable effect on both MMP-9 and TIMP-1 levels (231-233). Heparin has been shown to affect the concentration of TIMP-2 (234), increasing the concentration of TIMP-2 according to the heparin concentration. However, no similar effect was seen when looking at MMP-9 concentrations (234). It would not seem to be an unreasonable assumption that a similar effect could be seen on the concentration of TIMP-1. As the plasma samples in this study were taken with lithium heparin as the anticoagulant, the decision was therefore made to measure serum, rather than plasma, MMP-9:TIMP-1 ratios.

Serum MMP-9 and TIMP-1 levels were measured using commercially available ELISAs (R+D Systems; Minneapolis, USA) on serum samples that had frozen within 3 hours of collection, and that had not

been previously thawed. Both ELISAs were performed according to the manufacturers' instructions. MMP-9 concentrations were then expressed as a ratio to TIMP-1 concentration for each individual, in order to give an estimation of active MMP-9.

4.2.13. Immunophenotyping

Previously separated peripheral blood mononuclear cells were stored in liquid nitrogen, before being thawed, stimulated, stained and analysed towards the end of the study. The samples for analysis were selected according to the MS risk score derived for each individual. All analysis was performed blinded to the risk score associated with the individual whose samples were being analysed. 8 participants with MS, 8 siblings with high MS risk scores, 8 siblings with low MS risk scores and 8 healthy controls were selected for analysis in this sub-study.

Intracellular cytokine staining was performed for IL-17 and FoxP3. In addition, an aliquot of the cell culture supernatants of stimulated cells was removed after 5 hours of cell stimulation, stored at -80^{o}c, and IL-17A concentrations in the culture supernatant determined.

4.2.13.1. Cell stimulation

Cells were retrieved from the liquid nitrogen cryostore and stimulated on the day of thawing. During the thawing process, the temperature in the cryovial was rapidly increased to 30^{o}c, and the cryovial topped up with warm RPMI supplemented with 5% fetal calf serum (FCS). Over the next 2-3 minutes a further 4ml warm RPMI + 5% FCS was added to the cell suspension, prior to the more rapid addition (over approximately 30 seconds) of an additional 5ml RPMI + 5% FCS (to a total volume of 11ml). The cell suspension was then centrifuged at 330g for 10 minutes (with rapid acceleration and brakes on). The supernatant was aspirated, and the cells resuspended and washed in a further 10ml of warm complete medium.

The cells were then resuspended in 1ml RPMI + 5% FCS and counted with trypan blue using standard techniques. Following the counting procedure, cells were suspended in an appropriate volume of complete medium to give a final cell concentration of 2x10^{6} cells/ml.

5x10^{5} unsorted cells (0.25ml cell suspension) was then added to 0.75ml RMPI + 5% FCS (total volume 1ml) in a 24 well plate for cell stimulation. For each subject, one aliquot of cells was stimulated using PMA and ionomycin in the presence of golgistop, and one aliquot left unstimulated as a control. For the aliquot of cells to be stimulated, PMA was added to the suspended cells to achieve a final concentration of 50ng/ml, and ionomycin to achieve a final concentration of 1µg/ml. 1.6µl golgistop was also added to the 1ml cell suspension of stimulated cells. The cells were then incubated at 37^{o}c in the presence of 5% CO_2 for 5 hours in order to allow optimal stimulation to occur.

Following the five hour incubation, 250µl of the cell culture supernatant was removed and stored at -80^{o}c for future analysis. The cells were removed from the culture plates, with care taken to ensure that adherent cells were removed. The cells were washed with stain buffer (PBS + 5% FCS) and resuspended.

4.2.13.2. Cell surface and intracellular staining

The stimulated and unstimulated cells were fixed by adding 2ml BD Biosciences FoxP3 buffer A (catalogue no. 560098; BD Biosciences, Oxford, UK) to each aliquot and incubating for 15 minutes in the dark. Following fixation, cells were washed twice in stain buffer. The fixed cells were then stored overnight at 4^{o}c in the dark. Cell permeabilisation was performed using BD Biosciences Fox P3 buffer C (catalogue no. 560098; BD Biosciences, Oxford, UK). This was diluted according to the instructions supplied by the manufacturer, and 0.5ml added to each aliquot of cells. The cells were then incubated for 30 minutes in the dark.

Following permeabilisation, the fixed and permeabilised cells were washed and stained. Staining was performed by resuspending the cells in 150µl stain buffer prior to antibody addition. 5µl anti-human CD3-v450 (catalogue no 560365; BD Biosciences) and 5µl human Th17/Treg Phenotyping Cocktail (CD4 PerCP-Cy5.5, IL-17A PE, FoxP3 Alexa Fluor® 647) (catalogue no. 560762) were added to the suspended cells and incubated for 40 minutes in the dark.

The stained cells were then washed twice to remove excess antibody prior to analysis on the same day.

4.2.13.3. Flow cytometry

Flow cytometry analysis was performed on the BD Canto II flow cytometer in the flow cytometry core facility at the Blizard Institute. Prior to the initial data collection, appropriate compensation was performed with stained compensation beads using appropriate techniques.

Stained unstimulated and stimulated cells were analysed for each subject. Sample acquisition was gated to acquire data on $5x10^4$ CD3+/CD4+ cells. The difference in the proportion of cells detected as being positively stained for IL-17 and/or FoxP3 between the unstimulated and stimulated cells was used as the variable for analysis.

FlowJo v10.0.5 (Tree Star Inc., Ashland, OR USA) was used for analysis. Example plots are shown in figure 4.16. All analysis was performed blind to the disease state of the subject. The proportion of CD3+/CD4+ cells staining for either IL-17, Fox-P3 or demonstrating dual staining was calculated and recorded.

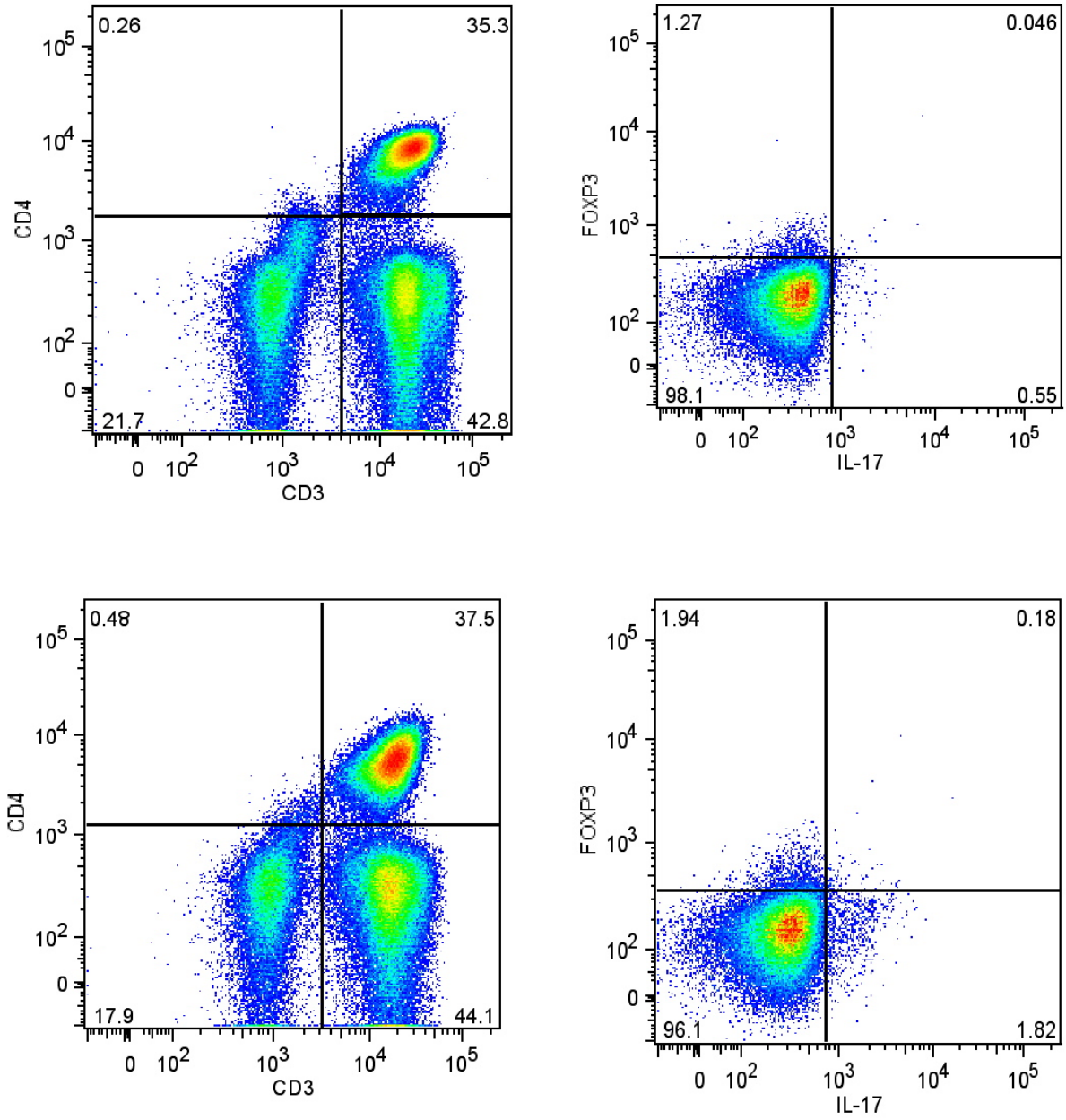


Figure 4.26: (a) Typical flow cytometry plot of unstimulated unsorted peripheral blood mononuclear cells. CD3+/CD4+ T cells can be seen in the upper right quadrant of the initial plot. These are then selected and the proportion of FoxP3 expressing, IL-17 expressing, or dual positive cells measured. **(b)** Typical flow cytometry plot of stimulated unsorted peripheral blood mononuclear cells from the same patient. The increase in the proportion of CD3+/CD4+ that express FoxP3, IL-17 or both can clearly be seen.

4.2.13.4. IL-17 measurement in cell culture supernatant

As a final step, IL-17A was measured in the cell culture supernatant of the stimulated cells. This was performed using the commercially available Quantikine human IL-17A ELISA (R+D systems; Abingdon, UK; catalogue number D1700). This ELISA was performed according to the manufacturers' instructions. All samples were assayed in duplicate.

4.2.14. MRI

4.2.14.1. Scan Acquisition

All MR imaging was performed at the NMR Research Unit, Institute of Neurology, Queen Square. Scanning was performed on a Philips Achieva 3 Tesla (T) MRI system with the manufacturer's product 32-channel head coil.

Images for lesion assessment were performed by acquiring PD/T2-weighted images using a 2D turbo spin echo (2D-TSE) sequence with field of view 240 x 180mm, voxel size 1 x 1x 3mm over 50 slices. Echo train length (ETL) = 10, repetition time (TR) = 3500ms, echo time (TE) = 19/85ms, number of excitations (NEX) = 1 and the scan time = 4 minutes. Other images were obtained during the MRI acquisition, however these were not used to determine the presence or absence of lesions in keeping with demyelination.

4.2.1.4.2. Image interpretation

All image interpretation was performed by assessors blinded to the risk status of participants. All images were independently assessed by a trained neuroradiologist (Dr Paul Smith) and a neurologist trained in image interpretation in the context of MS (Dr Klaus Schmierer). Images were assessed for the presence of T2 hyperintensities in keeping with demyelination. Assessors were aware of the age and gender of the participants, but did not have any further details regarding the clinical status or MS risk profile of any of the participants. In cases of dispute between the two independent assessors, a third assessor, a consultant neuroradiologist (Dr Jane Evanson) provided a further independent opinion.

MRI scans were examined for the presence of T2 hyperintense lesions. Where present, lesions were assessed and a decision made whether they were likely to represent demyelination, alternative pathology or non-specific T2 hyperintensities using internationally accepted guidelines developed by MAGIMS (235, 236). Lesions were identified on T2 FLAIR weighted images, corroborated by T2 weighted and proton density images; very occasionally sagittal Phase Sensitive Inversion Recovery (PSIR) was used when the location of the lesion (periventricular/juxtacortical) was in question.

The presence, absence and total number of any T2 weighted hyperintensities was recorded, together with the location. A decision as to whether these lesions represented “possible demyelination” or “likely demyelination” was then made according to the above criteria (235, 236).

4.3. Methods 2: calculating an overall risk score

4.3.1. Calculating the relative risk associated with each risk factor

4.3.1.4. Gender

4.3.1.4.1. Background

Females have an increased risk of MS compared to males. There is no recent large-scale analysis defining the sex-specific relative risk (RR) of MS associated with being female. Whilst there has been much interest over the years in developing markers that predict development of CDMS following a CIS, there has been little work examining the effect of gender on this development. Many observational studies state the number of participants of each sex at study enrolment. I used this data to produce a relative risk according to gender for developing MS, CIS, and progressing from CIS to CDMS.

4.3.1.4.2. Search strategy

PubMed was hand-searched using the terms “multiple sclerosis AND prevalence”, “clinically isolated syndrome”, “multiple sclerosis AND conversion”, “first demyelinating event” and “first demyelinating event AND conversion” on 1st August 2011. Abstracts and papers were then hand searched. Where the same cohort of patients was clearly used for repeated analysis, attempts were made where possible to use only the most recent studies in order to reduce population bias. Papers published between 2000 and 2011 were used. In order to examine sex ratio in radiologically isolated syndrome, RIS, PubMed was searched with the terms “radiologically AND isolated AND syndrome” or “RIS AND multiple AND sclerosis” or “RIS AND demyelination.

Only non-interventional studies were selected. Studies were included if they included unselected patients with either MS or a CIS (or both, but treated as separate groups), had greater than 10 participants, and provided data regarding the gender of participants. When examining the role of

gender in the development of CDMS following a CIS, the subgroup of non-interventional studies giving the number of males and females progressing to CDMS were selected. For those studies examining RIS, original observational accounts only were used. Papers used were limited to those that used accepted MRI criteria (Barkhof) for the radiological diagnosis.

All studies meeting these criteria were used to determine whether gender plays a role in the development of MS and CIS. Studies giving follow-up data with conversion rates to CDMS were used to calculate the effect of gender on progression to CDMS.

4.3.1.1.3. Statistical analysis

The meta-analysis was performed using RevMan 5.0 (Cochrane collaboration). A generic inverse variance model was used with a random effects model, as all I^2 values were >25%. In addition to the primary analysis, a conservative analysis was also performed, excluding studies where there was a reasonable degree of suspicion that patient cohorts overlapped between studies.

4.3.1.1.4. Included studies

Following the search for "multiple sclerosis AND prevalence", 4994 records were retrieved. Records published from 2000 onwards were hand searched. Those papers giving information on the prevalence of MS according to either the MacDonald or Poser criteria, and providing some information regarding gender were selected for inclusion. Where raw data regarding the number of males and females with MS were not provided, corresponding authors were contacted to provide data. 763 studies were retrieved from PubMed using the term "clinically isolated syndrome", 254 for "multiple sclerosis AND conversion" and 91 for the term "first demyelinating event". There was considerable overlap between results, and duplicate results were manually identified and removed.

69 studies meeting the inclusion criteria gave information regarding the sex ratio of MS (see figure 4.17 and appendix 5). 33 gave information regarding the sex ratio of CIS (see figure 4.18a and

appendix 5), 9 provided information regarding sex and the development of CDMS following a CIS (figure 4.19 and appendix 5), and 6 provided information on the sex ratio in RIS (figure 4.20 and appendix 5). For the conservative analysis, eighteen studies were excluded from the calculation of RR for females of MS and four studies were excluded from the CIS calculations. One of these studies was also excluded from the calculation of the RR of CDMS following CIS.

4.3.1.1.5. Risk of MS

69 studies were used to calculate the relative risk of MS according to sex (figure 4.17 and appendix 5). These studies yielded 71,849 patients with MS: 49,900 females and 21,280 males. The overall RR of MS in females was 2.22 (96% CI 2.10-2.34) compared to males (figure 4.17). There was significant between-study heterogeneity; I^2=93%, p<0.00001, but no clear evidence of publication bias on a funnel plot.

4.3.1.1.6. Risk of CIS

33 studies were used to calculate the RR of CIS according to sex (figure 4.18a and appendix 5). These yielded 4732 subjects with a CIS; 3248 female and 1466 males. The overall RR of CIS in females was 2.12 (95% CI 1.94-2.32) compared to males (figure 4.18a). There was significant between study heterogeneity; I^2=69%, p<0.00001. Effect sizes of individual studies appeared to be uniformly distributed around the meta-analysis effect size on a funnel plot, suggesting no large degree of publication or selection bias (figure 4.18b).

The majority of publications did not differentiate between forms of CIS. However, three publications selected patients with transverse myelitis as a presenting feature (237-239). When analysed separately to other studies these gave a RR in females of 2.96 (95% CI 1.72-5.10) compared to males. Similarly, 3 studies selected only patients presenting with optic neuritis (240-242). When analysed separately these gave a RR of optic neuritis as a CIS in females of 2.66 (95% CI 2.09-3.39) compared to males.

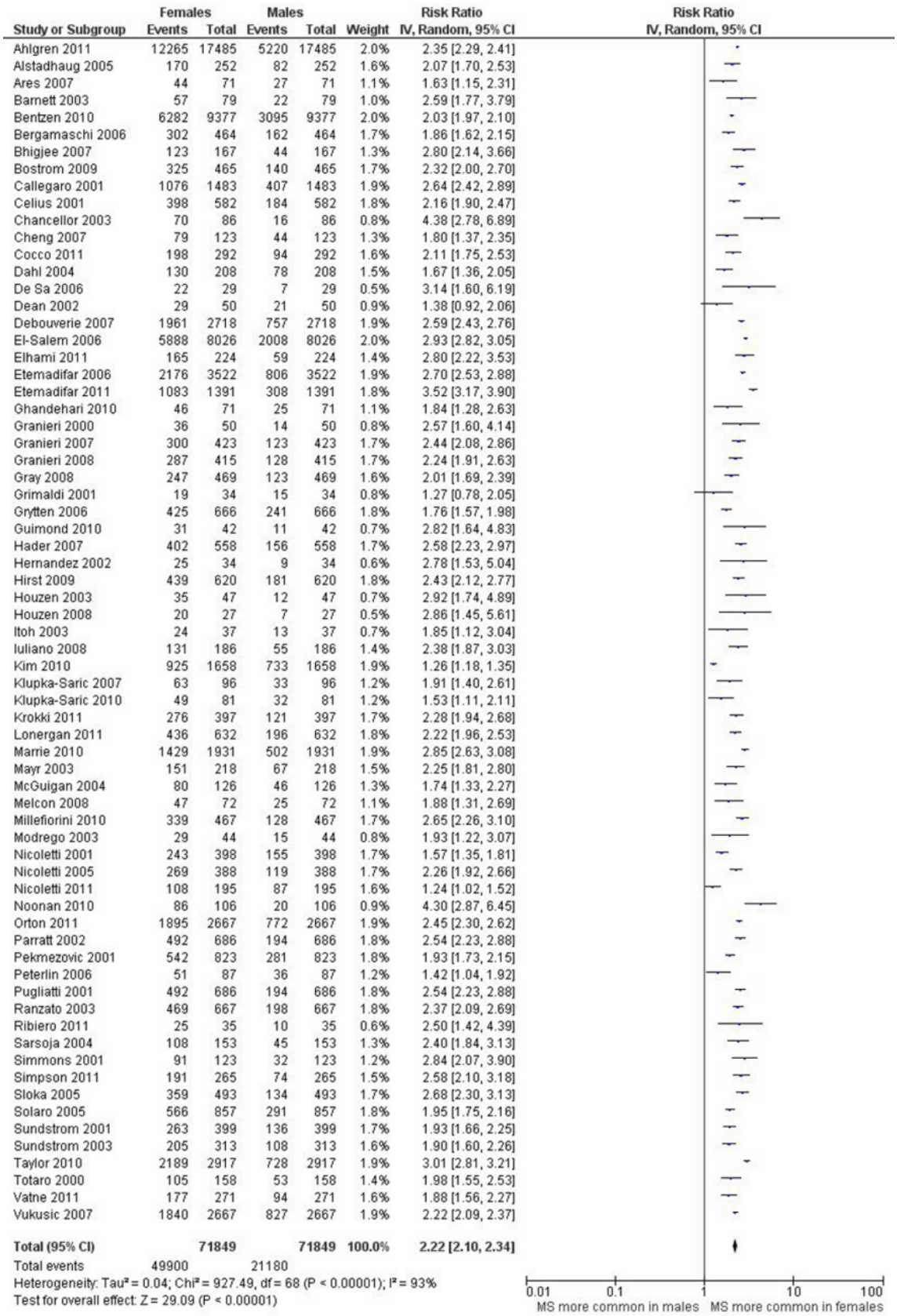


Figure 4.17: A meta-analysis of the sex ratio in MS from prevalence and observational studies 2000-2011

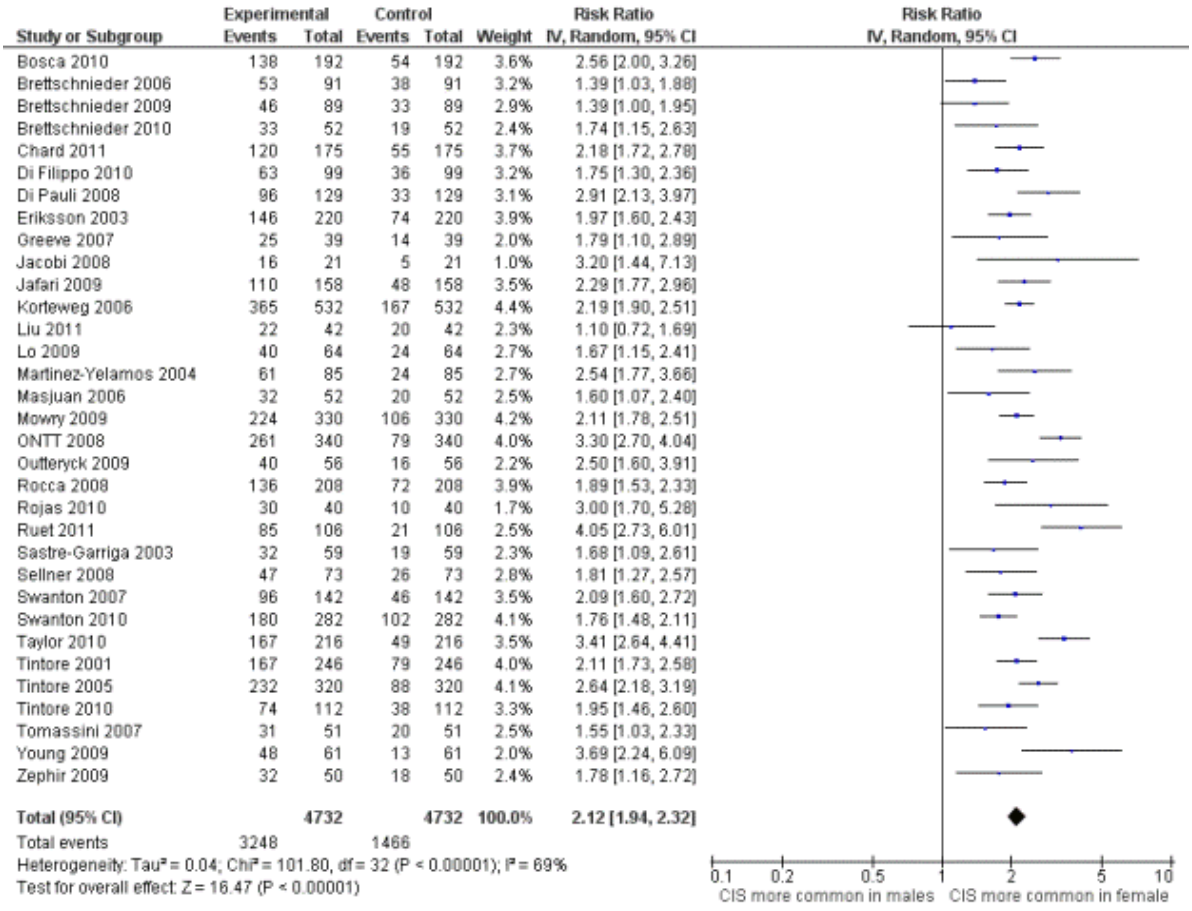


Figure 4.18: (a) A meta-analysis of the sex ratio in CIS.

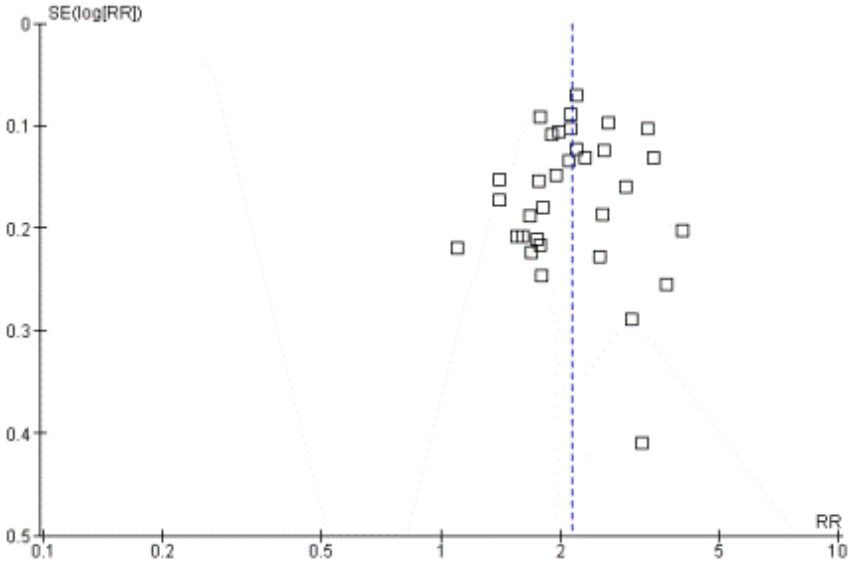


Figure 4.18: (b) Funnel plot of relative risk of CIS.

4.3.1.1.7. Risk of MS following a CIS

Nine studies (see figure 4.19 and appendix 5) were used to calculate the relative risk of progression to CDMS following a CIS according to sex. There were a total of 1116 subjects in these 9 studies. The mean follow-up time was 6.25 years (median 4 years, range 2-20 years). Of the 1116 subjects with a CIS, 557 developed CDMS; 413 of them female and 144 male. The RR of a female developing CDMS following a CIS was 1.20 (95% CI 0.98-1.46) compared to males (figure 4.19). There was moderate between-study heterogeneity: I^2=46%, p=0.06. Although one study appeared to give a much higher relative risk, this study was given little weight (1.0% in the generic inverse variance model), and so exclusion of this study did not significantly affect the overall result. There was no relationship between length of follow-up and RR associated with female sex.

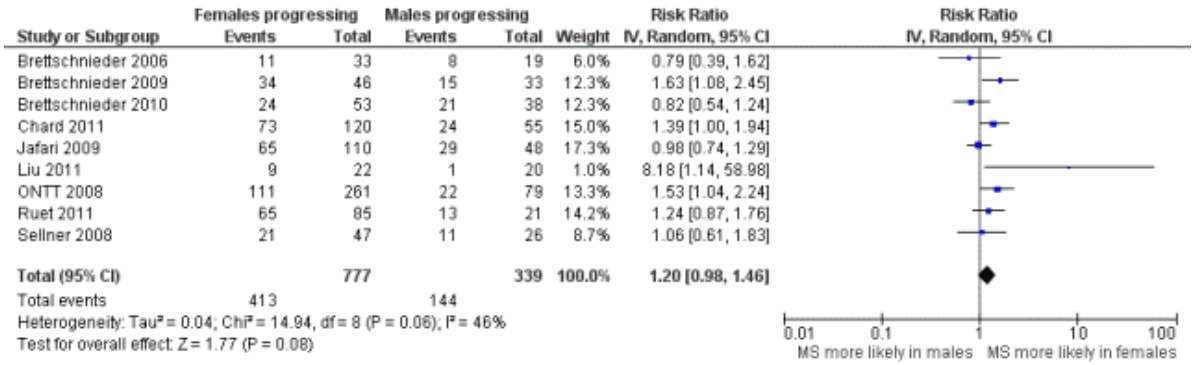


Figure 4.19: A meta-analysis of the relative risk of conversion from CIS to MS in females vs. males

4.3.1.1.8. Risk of radiologically isolated syndrome (RIS)

Six studies were included in the study of the relative risk of RIS in females (figure 4.20). This yielded a total of 224 subjects with RIS, of whom 177 were female. This gave a RR of 3.20 (95%CI 2.20-4.94) of RIS in females. The between-study heterogeneity was not judged to be significant in this analysis (I^2=48%, p=0.09), although the I^2 value supported the use of a random effects model.

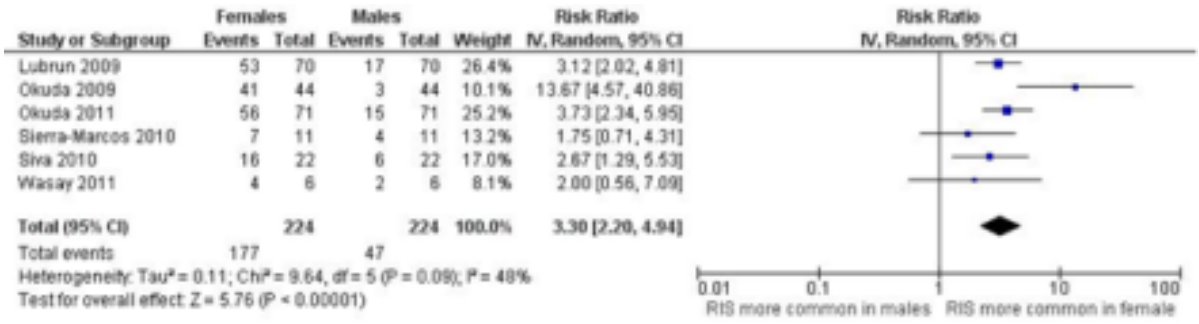


Figure 4.20: A meta-analysis of sex ratio in RIS from prevalence and observational studies 2000-2011

4.3.1.1.9. Conservative analysis

A conservative analysis of the studies used to calculate the RR of MS in females compared to males included 51 studies, with a total of 58,489 subjects (40,926 female, 17,423 male). This gave an overall RR of MS in females of 2.22 (95%CI 2.08-2.38) (data not shown). Between-study heterogeneity remained high with I^2=94%, p<0.00001. The conservative analysis of the CIS studies (3986 subjects with a CIS; 2750 female and 1228 males) gave an overall RR of CIS in females of 2.17 (95% CI 1.97-2.38) compared to males. Similarly to the initial analysis, there was significant between study heterogeneity; I^2=78% p<0.00001 (data not shown). In the conservative analysis, 489 patients developed CDMS, 368 of them female and 121 male. The RR of a female developing CDMS following a CIS in the conservative analysis was therefore 1.18 (95% CI 0.95-1.46). There was a degree of between study heterogeneity, I^2=45%, p=0.06.

4.3.1.1.10. Conclusions

It can therefore be seen that the RR of MS in females is 2.22 compared to males. This value is the same for both the overall and conservative analyses. This is similar to the RR of CIS in females compared to males; a fact that is supported by the non-significant effect of gender on conversion from CIS to MS seen in both the overall and the conservative analyses. It may be the relative risk of

RIS in females is even higher than MS or CIS, however the wide 95% confidence interval and small number of study subjects makes this data difficult to interpret.

4.3.1.2. Month of birth

4.3.1.2.1. Background

The "month of birth" effect, where those born in the winter appear to have a reduced MS risk and those born in the spring have an increased risk, has been interpreted as indicating a pre-natal role for vitamin D in modulating MS risk (243). The month of birth effect was first described in 1987 (244), and the first large-scale study by Willer et al (245) was performed in 2005. A number of studies examining the variation in MS risk associated with an individual's month of birth have been performed. These have enrolled varying numbers of participants, although more recently national MS registers have facilitated large-scale population-based studies (245-248).

Studies have been performed at a range of latitudes, meaning that there is a large inter-study variation in the change in UV light exposure between seasons. At latitudes greater than approximately 52^{o} from the equator, insufficient UV light of the correct wavelength (UVB; 290-315 nm) reaches the skin between October and March to enable vitamin D synthesis during the winter months (249). It would therefore be expected that studies examining those populations living at latitudes greater than 52^{o} would demonstrate a significant month of birth effect, whereas those at lower latitudes would not. However, no large-scale study has studied this potential variation to date. I therefore set out to review and integrate the existing data on month of birth and subsequent MS risk by performing a systematic review and meta-analysis.

4.3.1.2.2. Search strategy and inclusion criteria

PubMed and Web of Science were searched using the terms "multiple sclerosis" AND "month of birth", "multiple sclerosis" AND "month" and "multiple sclerosis" AND "season". Papers were then evaluated using the inclusion criteria described below. Additionally, the references of evaluated articles were screened for additional publications meeting the inclusion criteria. The numbers of

observed and expected births for MS patients and healthy controls in each month were recorded for each dataset. The odds ratio was then calculated using the observed and expected MS birth rate for each month.

Where seasonal data only were given, the months used to define each season were examined. Prior to performing the search, the decision had been taken to use the UK Met Office definition of each season (250): winter: December, January, February; spring: March, April, May; summer: June, July, August; autumn: September, October, November. Papers that provided seasonal data not adhering to these categories (251, 252) were excluded at this point. Data given according to month of birth were combined for seasonal analysis. Only one study gave data purely by season of birth (253).

Inclusion criteria were pre-specified. Papers had to be published after 2000, include both MS and healthy control (HC) groups, and provide either month or season of birth data for each population. The relative risk of MS for each month of birth had to be described compared to a HC population, rather than relative to a reference month.

4.3.1.2.3. Statistical analysis

Review Manager 5.1 (Cochrane collaboration) was used for the initial statistical analysis. The generic inverse variance model with fixed effects was used (random effects model used only when I^2>25%) to compare the observed and expected MS births in each month and season.

4.3.1.2.4. Included studies

The initial search generated 38 results. Three papers were excluded as they did not include a control group, two papers calculated risk compared to a reference month rather than a reference population, and two papers gave seasonal analysis using a different definition of seasons to the pre-specified definition. The details of the screening and inclusion process are given in figure 4.21. The ten remaining papers were considered for inclusion in the analysis (245-248, 253-258). Details of the included papers are given in table 4.6. A total of 172,918 MS births were identified, of which 151,978

were included in the analysis. There was little significant heterogeneity in any of the analyses (I^2 range 0-91%, with 6 months having an I^2 of 0% for at least one of the analyses performed).

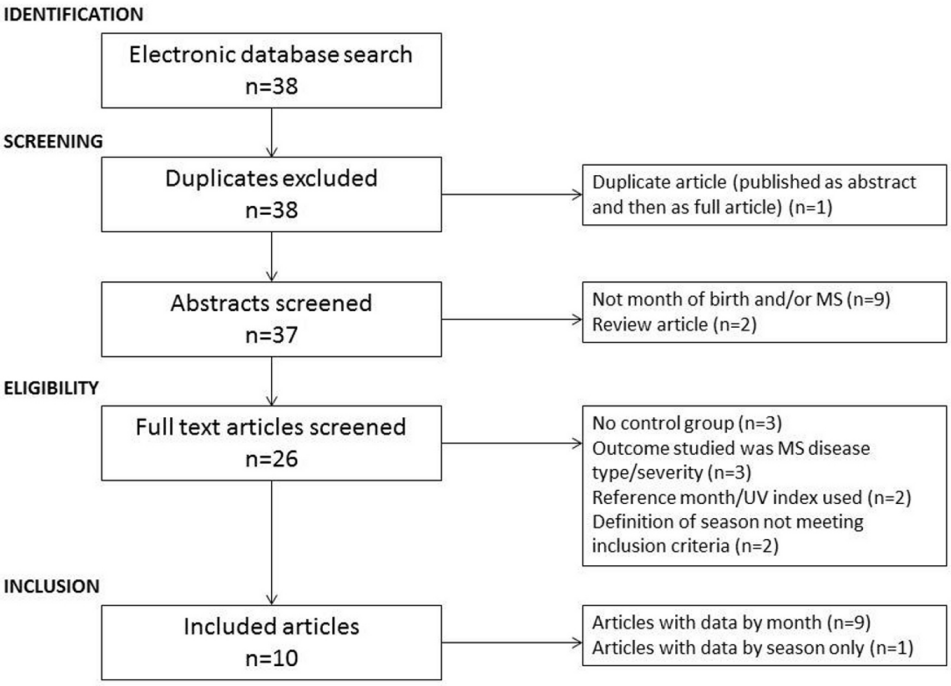


Figure 4.21: Screening and inclusion process for studies included in month of birth analysis

In the initial analysis the paper by Sadovnick et al (257) was excluded, as the authors state that they use an identical dataset to that used by Willer et al (245). Additionally the paper by Ramagopalan et al (256) was excluded, as the population used in this paper was mostly encompassed by that studied by Willer et al (245) and Salzer et al (255).

A population-conservative analysis excluded additional papers where there was a reasonable suspicion that duplicate populations were being examined. Papers excluded in this analysis were those by Willer et al (245) (UK data), Ramagopalan et al, Sadovnick et al and Bayes et al (253, 256,

257). In each case, in order to ensure maximum case ascertainment, the paper citing the highest number of cases was retained and all others excluded.

Table 4.6: included studies

Author and year	Country (region)	Estimated latitude	Total MS (n)	Months associated with an excess risk of MS (OR; 95% CI)	Months associated with a lower risk of MS (OR; 95% CI)
Disanto 2012 (248)	UK	53.1°N	26,994	April (1.05; 1.002-1.09) May (1.08; 1.04-1.13) June (1.04; 1.01-1.09)	October (0.96; 0.92-1.00) November (0.96; 0.91-1.00)
Menni 2012 (247)	Italy (Milan, Pavia, Sardinia)	45.2°N	2,737	April (1.17; 1.02-1.32)	October (0.88; 0.76-0.99)
	Denmark	55.7°N	15,900	Nil	November (0.94; 0.89-1.00)
	USA Caucasians	Excluded	50,650	June (1.06; 1.03-1.09) July (1.04; 1.01-1.07)	January (0.97; 0.94-1.00) February (0.97; 0.94-1.00) October (0.97; 0.94-1.00)
	USA African Americans	Excluded	5370	July (1.11; 1.01-1.21)	Nil
Givon 2012 (254)	Israel (Tel Hashomer)	32.3°N	2,264	Nil	December (0.84; 0.71-0.96)
Saastamoinen 2011 (246)	Finland (Helsinki)	62.4°N	8,359	April (1.09; 1.01-1.17)	January (0.91; 0.84-0.98) November (0.89; 0.82-0.96)
Bayes 2010 (253)	Scotland (Glasgow)	56.8°N	1,309	Spring (March, April, May) (1.17; 1.05-1.29)	Autumn (September, October, November) (0.87; 0.77-0.98)
Salzer 2010 (255)	Sweden (Umea)	59.3°N	9,361	June (1.11; 1.03–1.19)	January (0.90; 0.84–0.98) December (0.92; 0.85–1.00)
Ramagopalan 2009 (256)	Canada, Norway and Sweden	Excluded	4,834	Nil	Nil
Sadovnick 2007 (257)	Canada	56.8°N	14,799	June (0.95; 0.90-1.00)	November (0.90; 0.84-0.95)
Willer 2005 (245)	Canada	56.8°N	17,874	Nil	November (0.91; 0.85-0.97)
	UK	53.1°N	11,502	May (1.18; 1.10-1.26)	November (0.89; 0.81-0.98) December (0.91; 0.83-0.99)
Salemi 2000 (258)	Sicily	37.6°N	965	Nil	Nil

A geographically-conservative analysis examined the effect of month of birth in those populations with a clear and consistent difference in UV radiation between months. This analysis selected those studies where the latitude associated with the population was greater than 52°. Included papers were those by Menni et al (247) (Danish data only), Willer et al (245), Saastamoinen et al (246), Disanto et al (248), and Salzer et al (255). Ramagopalan et al (256) was excluded from this analysis, as it was not possible to estimate the latitude of the population, as samples from three countries

were used in the study. Additionally, Sadovnick et al (257) was excluded from this analysis as the authors state that the dataset used was previously used by Willer et al (245). The figure of 52^{o} was chosen because at latitudes of about 52^{o} and above, there is no UV light of appropriate wavelength for the cutaneous synthesis of vitamin D during October – March (249). People living at these latitudes would therefore be expected to have significant variation in vitamin D levels over the course of a normal year.

Finally, an overall-conservative analysis was performed where all of the studies that were excluded in the population- and geographically-conservative analyses were excluded. Papers included in this section of the analysis were Menni et al (247) (Danish data only), Willer et al (245) (Canadian data only), Saastamoinen et al (246), Disanto et al (248), and Salzer et al (255).

4.3.1.2.5. Month and season of birth

Nine studies gave information on month of birth and subsequent MS risk (245-248, 254-258). The studies by Ramagopalan et al (256) and Sadovnick et al (257) were excluded from this analysis for the reasons described in the methods above. The O:E ratio of MS by month of birth is given in table 4.7. When all studies were included there were significantly fewer observed MS births than expected in October (O:E=0.95, p=0.04), and November (O:E=0.92, p=0.01). There were significantly more MS births than expected in April (O:E=1.05, p=0.05) (table 4.7 and figure 4.22a). There was a significant variation in MS births when looking at seasonal data (figure 4.23a).

Table 4.7: Observed: expected MS cases by month of birth. *p*-values are given for significant results in bold, and for borderline significant results (not bold).

Analysis	Observed:expected MS births (ratio (95% CI); significance)											
	January	**February**	**March**	**April**	**May**	**June**	**July**	**August**	**September**	**October**	**November**	**December**
All studies (n=151,978)	0.96 (0.92-1.01)	0.98 (0.94-1.01)	0.99 (0.95-1.03)	1.05 (1.00-1.11) **p=0.05**	1.05 (0.98-1.13)	1.05 (0.93-1.19)	1.03 (0.98-1.08)	1.01 (0.98-1.05)	0.98 (0.95-1.01)	0.95 (0.91-1.00) **p=0.04**	0.92 (0.86-0.98) **p=0.01**	0.98 (0.96-1.01)
Population conservative (n=140,474)	0.95 (0.91-1.00) p=0.07	0.98 (0.04-1.02)	0.99 (0.95-1.04)	1.05 (0.99-1.12) p=0.08	1.03 (0.98-1.09)	1.05 (0.91-1.02)	1.02 (0.97-1.08)	1.01 (0.98-1.05)	0.98 (0.95-1.02)	0.96 (0.91-1.01)	0.93 (0.87-1.00) **p=0.04**	0.98 (0.93-1.03)
Geographically conservative (n=89,059)	0.95 (0.88-1.02)	1.01 (0.96-1.06)	0.99 (0.94-1.04)	1.08 (1.03-1.12) **p=0.001**	1.11 (1.03-1.21) **p=0.007**	1.06 (1.00-1.13) **p=0.05**	1.01 (0.97-1.06)	0.99 (0.94-1.04)	0.96 (0.92-1.01)	0.94 (0.89-0.98) **p=0.006**	0.89 (0.82-0.96) **p=0.004**	0.97 (0.90-1.04)
Overall conservative (n=78,488)	0.93 (0.86-1.01)	1.01 (0.96-1.06)	0.99 (0.93-1.06)	1.08 (1.02-1.13) **p=0.004**	1.09 (1.03-1.14) **p=0.002**	1.06 (0.99-1.14)	1.00 (0.96-1.05)	0.99 (0.94-1.05)	0.97 (0.92-1.01)	0.95 (0.90-1.00) **p=0.03**	0.90 (0.82-0.99) **p=0.03**	0.99 (0.93-1.06)

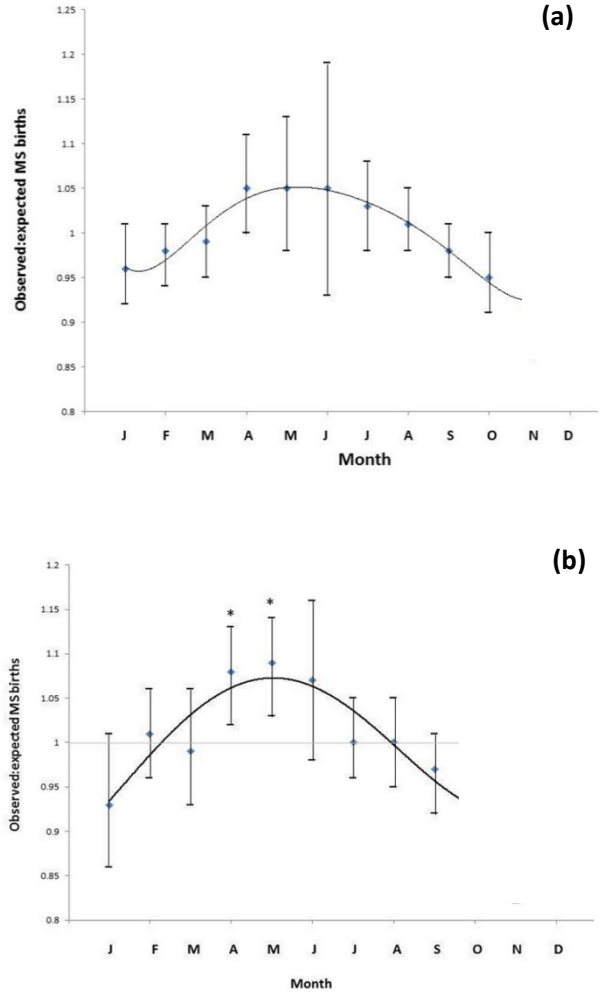


Figure 4.22: (a) Variation in observed: expected MS births over the year calculated using all studies. The points represent the absolute values and the bars the 95% CI.

(b) Variation in observed: expected MS births over the year calculated using the overall-conservative selection strategy. The points represent the absolute values and the bars the 95% CI.

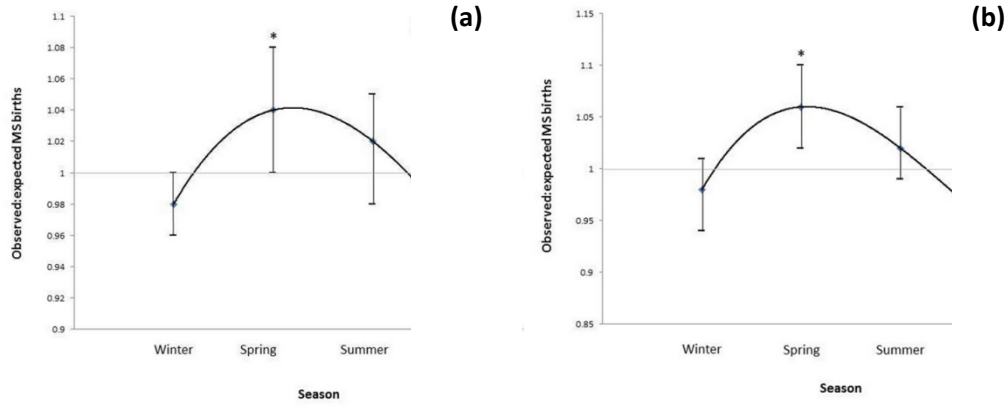


Figure 4.23: (a) Variation in observed: expected MS births between seasons calculated using all available studies. The points represent the absolute values and the bars the 95% CI. **(b)** Variation in observed: expected MS births between seasons calculated using the overall-conservative selection strategy. The points represent the absolute values and the bars the 95% CI.

4.3.1.2.6. Conservative analyses

When the population-conservative analysis was performed, much of the significance was lost. The only month in which there was a significant deviation of MS births from expected was November, where there were significantly fewer MS births than expected (O:E=0.93, p=0.04) (table 4.7).

When the geographically-conservative analysis was performed, the effect of UV variation over the course of the year was highlighted. There were significantly more observed MS births in April than expected (O:E=1.08, p=0.001), May (O:E 1.11, p=0.007) and June (O:E=1.06, p=0.05), and significantly fewer in October (O:E=0.94, p=0.006) and November (O:E=0.89, p=0.004) (table 4.7). Conversely, when only those studies performed at <52^{o}N were selected (Menni et al (Italian data) (247), Givon et al (254)and Salemi et al (258)), the month of birth effect was lost, bar a borderline significant increase in observed MS births in June (O:E=1.21, 95% CI 1.01-1.44, p=0.04).

Finally, the overall-conservative analysis was performed (cases=78,488). In this analysis there were significantly more observed MS births in April than expected (O:E 1.08, p=0.004) and May (O:E 1.09, p=0.002), and significantly fewer in October (O:E 0.95, p=0.03) and November (O:E 0.90, p=0.03) (table 4.7 and figure 4.22b). There was an increase in observed MS births in spring and a decrease in autumn (figure 4.23b).

4.3.1.2.7. Conclusions

It can therefore be seen that month of birth has a significant effect on subsequent MS risk. As the population included in the sibling study were almost entirely born in the UK, the associated risks generated by the population and geographically conservative analysis are the most appropriate to be used in the final analysis.

Through combining existing datasets for month of birth and subsequent MS risk, this provides the most robust evidence to date that the month of birth effect is a genuine one. Whilst this has previously been shown in a number of studies, many of these studies have used similar or

overlapping datasets, such as that developed by the Canadian Collaborative Project on the Genetic Susceptibility to Multiple Sclerosis (CCPGSMS), used by (245, 256, 257). Whilst the latitudinal data supports the month of birth effect being a result of UVB (and hence vitamin D) variation, it could result from any factor that varies in a similar seasonal and latitudinal manner. It must be noted that there is a large body of evidence supporting the importance of vitamin D in multiple sclerosis (259). Maternal vitamin D levels are one possible explanation for the month of birth effect, however this hypothesis requires further investigation, as this is only one facet through which vitamin D may exert an effect.

The conservative analysis, which excludes studies where there is evidence that the patient data used may be either wholly or partially overlapping, ensured that effects exclusive to individual datasets were not subject to magnification in the overall analysis. In the population-conservative analysis, the month of birth effect was lost in all months bar November. This is likely to be due to the fact that the excluded studies were all performed at a latitude >52^{o}N, and so the UV variation over the course of a year was significantly reduced in the remaining studies.

When the studies that were performed at a latitude of <52^{o}N were excluded, the month of birth effect once again became apparent. There was a highly significant increase in MS births in both April and May, and a reduction in October and November. Only one study (248) has previously shown a significant reduction in all of these months. This finding was complemented by the demonstration that the month of birth effect is almost entirely lost when selecting those studies performed at <52^{o}N. However, it must be noted that the geographically conservative analysis has the potential to overestimate some population-specific risks, due to the high probability of duplicate data in the analysis. Any deviation present in these datasets will therefore be exaggerated in this analysis.

No studies from the Southern hemisphere were included in this analysis. This was not a deliberate selection criterion, but instead reflects the imbalance in the origin of such studies. Whilst studies into the month of birth effect in the Southern hemisphere do exist; including Staples et al.,

(Australia) (260) and Fragoso et al., (Brazil) (261); the data was not presented in a manner that could be used in this analysis. There remains a need for further studies in the Southern hemisphere in order to confirm if the reversal in the month of birth effect noted by Staples et al., (260) exists in other countries.

The overall conservative analysis had the least number of participants, 78,488. However, it is likely to be the most appropriate analysis, as both potential duplicate datasets and those studies performed in areas with low variation in UV exposure during the year were excluded. The highly significant increase in MS risk in those born in April and May remains clear, as does the reduction in risk in those born in October and November. By pooling data and performing a meta-analysis, the month of birth effect can be extended from that previously described. This effect is highlighted when looking at the differences in MS risk stratified by season of birth.

However, it must be borne in mind that this is a meta-analysis of existing data, and therefore has weaknesses in keeping with this methodology. Publication and selection bias are potential problems, although studies showing both no effect and significant effects were included, and there was no evidence of bias in funnel plots. Additionally, although steps were taken to attempt to exclude duplicate data, it may be that some remains, influencing the results. The lack of Southern hemisphere studies is a significant limitation, as a demonstration of the reversal of the month of birth effect would strengthen confidence in this finding considerably. Combining the data from individual months into seasons (as defined by the UK Met Office) may have introduced bias into the results, either strengthening or weakening the association between season of birth and MS. However, combining the data in this way allowed the addition of a further study. These results must therefore be taken in the context of the other results presented here.

A recent study (369) has criticised the existing body of work on month of birth, stating that the effect seen was a false effect caused by confounding due to poor matching of cases and controls. The majority of month of birth studies compare the distribution of cases to controls drawn from general

population registers, not necessarily matched for year of birth. Fiddes et al (369) argue that temporal variations in month of birth underly the effect in the MS population. However, when the pooled group of controls used in this analysis were compared to an assumption of a uniform birth rate, a clear variation in month of birth was seen in the controls, with the same pattern as that seen in MS. This implies that the effect seen in MS is over and above that seen in controls. In addition, Fiddes et al ignore the fact that the Australian study matched cases to controls with rigorous criteria, including matching by year of birth.

In conclusion, this work, which uses the largest number of patients to date, confirms and extends the month of birth effect seen in MS. Through the demonstration of a relationship between month of birth effect magnitude and latitude, and hence a possible interaction, it supports ambient UV radiation as a possible pre-natal environmental modulator of MS risk.

4.3.2. Other relative risks taken from the literature

4.3.2.1. Infectious mononucleosis

The relative risk of those with a previous history of infectious mononucleosis (IM) (symptomatic primary infection with Epstein-Barr virus) has recently been determined in an updated meta-analysis (262). By examining 19390 reported cases, the authors were able to demonstrate a strong relationship between IM and subsequent MS risk, with a relative risk of 2.17 (95% CI 1.97-2.39) (262).

4.3.2.2. Titre of IgG directed against Epstein-Barr virus Nuclear Antigen-1 (EBNA-1)

There are two facets to the risk of MS associated with the titre of IgG directed against the Epstein-Barr virus nuclear antigen-1 (EBNA-1) – the reduction in risk associated with negative antibody status, and the increasing risk associated with higher titres of IgG (133). The single largest study examining this used a nested case-control study design examining 3 million US army recruits, 83 of whom subsequently developed MS. Anti-EBNA-1 IgG titres were divided into quintiles, and the relative risk of MS associated with each quintile calculated (133). The relative risk associated with each quintile was related to quintile 1 (the lowest quintile); these are described in table 4.8. As indirect immunofluorescence was used in the index publication, the cut-off values for the quintiles could not be directly transferred to this analysis, where ELISA was used to assess IgG titre. Quintile cut-offs were therefore generated from the healthy control and sibling population being studied.

Table 4.8: The relative risk associated with each quintile of antibody titre of IgG against EBNA-1. Values taken from Levin et al 2005 (133) unless otherwise stated.

Quintile	Odds ratio (95%CI)
Negative (130)	0.33 (0.22-0.50)
Q1 (lowest)	1.0 (reference quintile)
Q2	2.6 (0.7-9.2)
Q3	3.2 (1.0-10.4)
Q4	5.1 (1.5-17.6)
Q5	9.4 (2.5-35.4)

Undetectable/negative IgG titres against EBNA-1 are also a significant modulator of MS risk. A recent meta-analysis examined the odds ratio of MS associated with EBNA-1 negativity, as measured by both indirect immunofluorescence and ELISA (130). The overall OR associated with EBNA-1 negativity was 0.18 (95% CI 0.13-0.26). However, when only those studies using ELISA were selected the OR of MS increased to 0.33 (95% CI 0.22-0.50) (130). As this study used ELISA to determine anti-EBNA-1 IgG titres, an OR of 0.33 was used in the calculation of the overall risk score.

4.3.2.3. Serum 25-hydroxyvitamin D

Low serum 25-OHvD levels have been associated with an increased risk of MS (147). A prospective, case-control nested study of US army recruits demonstrated a change in odds ratio for the subsequent development of MS of 0.59 (95% CI 0.3-0.97) for every 50nmol increase in serum 25-OHvD level. The cohort were also divided into quintiles according to serum 25-OHvD levels, and the OR of developing MS for each quintile calculated with the lowest quintile as the reference (147). As the serum levels of the population studied were higher than those of the population in this study, the quintile cut-offs specified by Munger et al (147) were used to define quintiles (see table 4.9).

Table 4.9: Quintile cut-offs and relative risks associated with serum levels of 25-OHvD. Values taken from Ascherio et al (147)

Quintile	Odds ratio (95% CI)
Q1 (lowest; 15.2-63.2 nmol/l)	1.0 (reference quintile)
Q2 (63.3-75.3 nmol/l)	0.57 (0.3-1.07)
Q3 (75.4-84.8 nmol/l)	0.57 (0.3-1.07)
Q4 (84.9-99.1 nmol/l)	0.74 (0.40-1.36)
Q5 (99.2-152.9 nmol/l)	0.38 (0.19-0.75)

4.3.2.4. Smoking status

A recent meta-analysis examined the risk of MS associated with “ever and/or current smoking”. A relative risk of 1.52 (95% CI 1.39–1.66) was associated with smoking prior to disease onset (i.e. “ever smoking” (154).

4.3.2.5. HLA-DRB1*1501 haplotype

In the recent genome-wide association study, possession of the HLA-DRB1*1501 haplotype was associated with a relative risk of 3.1 (95% CI 2.9-3.3) of developing MS (103). This relative risk relates to those who are heterozygotes for HLA-DRB1*1501. Homozygosity for the HLA-DRB1*1501 allele has been associated with a higher risk, and so the relative risk was doubled in homozygotes (157).

4.3.2.6. SNPs identified by GWAS

The recent GWAS identified 4 HLA and 57 non-HLA SNPs that appear to contribute to MS risk, albeit each conferring a small relative risk. These are detailed in tables 4.4 and 4.5 on page 109 and 110. The relative risk associated with each SNP given in the GWAS refers to a heterozygote for the risk SNP, with a doubling of the risk in homozygotes.

4.3.3. Interaction between risk factors

4.3.3.1. Existing evidence

Risk factor interaction in multiple sclerosis remains an area of interest to many researchers. Results published to date have been contradictory. Particular areas of interest have surrounded the interaction between combinations of smoking, HLA-DRB1*1501 haplotype and anti-EBNA-1 IgG titre. In order to detect interactions between factors with relatively small effect sizes in a relatively rare illness such as MS, studies of supra-population size are required. Large-scale population-based studies have only recently become feasible; however to gain adequate power to fully examine potential interactions international collaborations are needed. There are some indications of possible risk factor interactions emerging from rigorous population-based studies, however these remain at risk of type 2 statistical error due to underpowering.

One group has demonstrated a potential interaction between HLA-DRB1*1501 haplotype and anti-EBNA-1 IgG titre in terms of modulating MS risk. Sundstrom et al (263) demonstrated that in a case control study of 109 MS patients and 212 controls, HLA-DRB1*1501 positive controls had, on average, higher anti-EBNA-1 IgG titres than HLA-DRB1*1501 negative controls. There appeared to be an additional interaction in those with mid-range anti-EBNA-1 IgG titres; there was an increased risk of MS in those with titres in this range who were HLA-DRB1*1501 positive but not in those who were HLA-DRB1*1501 negative. They were also able to demonstrate some interaction in a larger study (264), with an interaction present on an additive scale but not on a multiplicative scale. This interaction was found to be present when IgG titres against the EBNA-1 fragment 385-420 were studied (156), with IgG directed against this fragment appearing to be a risk factor independent of conventionally measured anti-EBNA-1 IgG titres. However, these findings have not been replicated by other groups. Simon et al (265) did not find any interaction between anti-EBNA-1 IgG titre and smoking when studying 442 MS cases and 865 controls.

Similarly conflicting results exist when looking for any interaction between smoking and HLA-DRB1*1501 haplotype. Neither Simon et al not Sundqvist et al were able to demonstrate any interaction (264, 265). However, Hedstrom et al (158) were able to demonstrate an interaction between smoking and HLA-DRB1*1501 haplotype, albeit only in those subjects lacking the protective HLA allele, HLA-A*02.

A potential interaction between smoking and anti-EBNA-1 IgG titres appears to have a plausible biological basis. EBV activation and nicotine metabolism have been shown to share molecular pathways, including Jun-c-kinase (266), MAPK (267, 268) and NF-kB (269, 270). Despite this, no interaction between these two MS risk factors has consistently been found. Simon et al were able to demonstrate an interaction, showing that the increased risk of MS associated with smoking was only present in those with higher anti-EBNA-1 IgG titres (265). This was not replicated by Sundqvist et al (264), who did not demonstrate any interaction on either a multiplicative or an additive scale.

Another area where there appears to be a biological basis for an interaction is the possible interaction between vitamin D and presence of the HLA-DRB1*1501 haplotype. It has been shown that there is a biologically active vitamin D response element (VDRE) in the promoter region of HLA-DRB1 (271). This VDRE acts in vitro to increase HLA-DRB1*1501 transcription in the presence of vitamin D, providing a functional mechanism for an interaction. However, the study of any potential interaction in vivo have been limited to date, but have not demonstrated a significant effect during life (272).

It may well be that this interaction is important pre-natally, something that is extremely difficult to demonstrate conclusively. Emerging evidence suggests that this may be the case. T-cell receptor excision circles (TRECS), which can be measured by PCR in T-cells isolated from cord blood, provide a measure of pre-natal thymic output (273). Individuals born in May show a significantly increased number of TRECs in CD4+ and CD8+ T-cells compared to those individuals born in November (273). There was a significant inverse correlation between the number of TRECs in CD4+ and CD8+ T-cells

and 25-OHvD levels in cord blood (Spearman rho -0.37; p=0.009) (273), however clearly this falls short of demonstrating a causal relationship. There is additional evidence that MS-associated genes are active in the T-cells of cord blood (274), highlighting the potential pre-natal influence of MS risk factors.

A finding that requires replication is a potential interaction between month of birth and HLA-DRB1*1501 haplotype. Ramagopalan et al (256) demonstrated that there were significantly fewer patients with MS born in November who carried the HLA-DRB1*1501 allele compared to patients not carrying the allele; conversely patients with MS carrying HLA-DRB1*1501 had a higher number of April births. The mechanism and effect of this possible interaction on MS risk remains to be determined.

4.3.3.2. Month of birth and latitude

Following on from the study of month and birth and subsequent MS risk described in section 4.3.1.2, I set out to interrogate the available data for any interaction between population latitude and the effect of month of birth on MS risk.

4.3.3.2.1. Methods

The search strategy and selection criteria for this study are described in section 4.3.1.2. In order to be included in the latitudinal analysis, papers had to provide information about the geographical location of the population studied. The geographical location of the population used in each of the included papers was extracted from the original paper. Google maps (www.maps.google.com) were then used to determine the latitude. In those papers where databases from a large geographical area (such as an entire country) were used to determine population characteristics, the mid-point latitude of the geographical area was used in the analysis. In those papers where two or more geographically distinct regions were studied (i.e. different countries/continents) (245, 247) and separate population figures were given for the distinct regions, these were analysed as separate datasets. The Italian cohort studied by Menni et al (247) originates from three separate areas of Italy, and in this case the latitude of the central region of the three was used in the analysis. The single paper (256) where the datasets from three countries were combined into a single analysis was excluded from the latitudinal analysis. In one paper (247), data were given covering the entire geographical area of the United States of America (USA). This data was excluded from the latitudinal analysis due to the large area covered – the latitude of the USA ranges from 18.5^{o}N (Hawaii) to 71.2^{o}N (Alaska). The papers by Sadovnick et al (257) and Ramagopalan et al (256) were excluded from this analysis, as the authors specified that they had used a dataset overlapping with that used by Willer et al (245).

A linear regression model was used for this analysis (PASW v18 (SPSS)). Observed:expected (O:E) MS births/month were regressed on the population latitude, which had been determined as described above. The dependent variable was O:E MS births/month, and the independent variable latitude, and the contribution of latitude to the equation *O:E ratio≈(latitude*X)+constant* was assessed for each month in turn using a linear regression model. An additional independent variable for sample size was then added to the model in order to assess whether this affected the results obtained.

4.3.3.2.2. Results

When linear regression was carried out using latitude as the predictor variable, a significant relationship was seen between latitude and the ratio of O:E MS births for December (p for latitude to predict O:E ratio=0.039). A borderline significant prediction p-value was seen for May (p=0.093) and August (p=0.076); however these were not significant when corrected for multiple testing with a Bonferroni correction.

When study size was added as an additional predictor variable into the model, the effect of latitude was lost. Given that the three southernmost studies were also the three smallest studies, it is therefore difficult to know whether the latitudinal interaction is a function of study size, or is indeed a genuine effect. It must also be taken into account when interpreting this data that latitude may not act as a linear variable in terms of its effect on month of birth, but there were insufficient studies available to investigate this.

Given that the majority of the population included in the sibling study were born in the UK, the interaction between latitude and month of birth was not felt to be a significant confounder in this study.

4.3.3.3. Interaction data from sibling study

Given the uncertainty currently regarding any potential interaction between risk factors, and the inconsistencies in the evidence, the decision was taken to examine the data gathered during the course of this study for any evidence of risk factor interaction. Should any interaction between the presence, absence, or magnitude of any two risk factors become apparent during this work then it can be argued that this is likely to have an influence in this population. If, however, there is no such evidence then it could be argued that the effect of any interaction, if present, is sufficiently small in this population as to have no significant effect on the overall risk score.

Interaction analysis was performed using a liner regression model where both risk factors were linear (serum 25-hydroxyvitamin D, IgG titre against EBNA-1) and a logistic regression model where one variable was binary (smoking status, HLA-DRB1*1501 haplotype) in PASW v18 (SPSS). Given that multiple serial analyses were being performed a Bonferroni correction was applied and α set at 0.0001.

There appeared to be a relationship between gender and serum 25-hydroxyvitamin D (p=0.006), but this was not significant at the Bonferroni corrected α. There was no other significant interaction that could be detected between any of the non-genetic risk factor variables in this cohort. There was no detectable interaction between HLA-DRB1*1501 haplotype and any of the other risk factor variables in this population.

Interaction testing was not performed for the genetic risk factors (other than the HLA-DRB1*1501 haplotype) in this cohort. Given the number of non-MHC SNPs tested, this study did not come close to having sufficient power to perform this analysis. Additionally, given the nature of the study, a significant proportion of this cohort consisted of subjects who were first-degree relatives, which had the potential to lead to significant confounding. In the GWAS from which the list of SNPs was taken

(103), extensive examination for linkage disequilibrium leading to interaction was performed. During the final analysis in the GWAS, on SNPs believed not to be in significant linkage disequilibrium with one another were selected, and therefore extensive testing in my relatively small population was not necessary.

4.3.4. Constructing an overall score

The overall risk score was constructed by pooling all of the available data regarding relative risks described above to create an overall "risk score". This score was not conceived of as an absolute score, instead an indication of the relative risk of MS in an individual. There have been previous attempts to generate such a score, the most extensive of which was that by de Jager et al (157).

This score, which was developed prior to the most recent GWAS in MS, attempted to assign individuals both a genetic risk score, and then to integrate selected environmental factors in order to improve the score. Using 16 SNPs (2 MHC, 14 non-MHC), the authors argued for a weighted risk score, as the relative risks associated with the SNPs selected showed considerable variability. They calculated their "weighed genetic risk score" by first calculating the weight assigned to each SNP, equal to the natural log of the OR for each allele. The authors then multiplied the number of risk alleles for each SNP by the weight for that SNP and then took the sum across the 16 SNPs (157). Using this technique, a ROC curve demonstrated an AUC of 0.637. This improved to 0.683 when the risks attributable to IgG titres against EBNA-1 and smoking status were included in the model (157).

A further analysis was performed by de Jager et al (157) by calculating risk score categories using the healthy control risk score distribution. Using seven categories defined by the distance from the mean risk score (category 4 encompassing the mean), a significant difference in MS risk according to category was demonstrated. When those individuals in category 7 (highest risk of MS) were compared to individuals in category 1 (lowest risk of MS), a 10.1 (95%CI 6.9-17.4) fold increase in the odds of having MS was seen.

This approach is supported by the recent GWAS in multiple sclerosis (103). In this work, the model for cumulative genetic risk was taken to be one in which risk increases multiplicatively with each

additional copy of the relevant allele (additive increase on the log-odds scale) within a logistic risk framework (103).

Other authors have genotyped SNPs within matched MS and HC populations, and then used these to calculate the OR associated with each SNP in that population prior to attempting to calculate a risk score (275). A potential problem with this is that the risks associated with the SNPs may be over-inflated in the population studied.

Given the readily available information provided by the recent MS GWAS (103), together with the relatively small sample size in the sibling study, the decision was taken to use the OR given in the GWAS, with the log-odds risk additive model proposed by de Jager et al (157). The weighting given to each of the non-genetic risk factors and possession of the HLA-DRB1*1501 allele is given in table 4.10. The contribution from the other HLA haplotypes and the non-HLA SNPs is given in tables 4.4 and 4.5 on pages 109 and 110.

Table 4.10: Summary of relative risks used in the calculation of an overall risk score. The risk associated with carriage of the HLA-DRB1*1501 haplotype is given; for the risks associated with other HLA haplotypes and non-MHC SNPs see tables 4.4 and 4.5.

Risk factor	Relative risk used in risk score calculation	Log value used in additive model
Gender	Female: 2.22	0.35
Month of birth	April: 1.08	0.03
	May: 1.09	0.04
	October: 0.95	-0.02
	November: 0.90	-0.05
Previous infectious mononucleosis	2.17	0.34
Quintile of IgG against EBNA-1	Undetectable titres: 0.33	-0.48
	Q3: 3.2	0.51
	Q4: 5.1	0.71
	Q5: 9.4	0.97
Quintile of serum 25-OHvD	Q5: 0.38	-0.42
Smoking status	Ever smoking: 1.52	0.18
HLA-DRB1*1501 haplotype	Heterozygote: 3.1	0.49
	Homozygote: 6.2	0.79

Chapter 5: Study population and MS risk score

5.1. Results

5.1.1. Study population

A total of 302 participants were enrolled in this study: 78 probands with MS, 121 of their unaffected siblings (including 6 monozygotic and 3 dizygotic twin pairs), and 103 healthy controls with no first or second-degree relatives with MS. Demographic details are given in table 5.1. Although there was no significant difference between the average age of patients and their unaffected siblings, there was a significant difference in the average age between both patients with MS and their siblings and healthy controls (see table 5.1), with healthy controls significantly younger than the other participants. However, the age range was similar between the three groups.

Table 5.1: Demographic details of study participants

	MS	Unaffected siblings	Healthy controls
Number	78	121	103
Age (mean; SD; range)	47.26 (11.74; 20-74)	47.24 (12.55; 18-75)	41.22 (11.33; 21-72)[a]
Gender (M:F; %F)	8:70 (89.7% F)	38:83 (68.6% F)[b]	33:70 (68.0% F)
Season of birth (n; %)	Winter: 19 (24.4%)	30 (24.8%)	26 (25.2%)
	Spring: 18 (23.1%)	30 (24.8%)	29 (28.2%)
	Summer: 21 (26.9%)	31 (25.6%)	25 (24.3%)
	Autumn: 20 (25.6%)	30 (24.8%)	23 (22.3%)
Type of MS (n; %)	RRMS: 45 (69.2%)		
	SPMS: 16 (20.5%)		
	PPMS: 7 (9.0%)		
Treatment (n; %)	37 (47.4%)[c]		
EDSS (mean; range)	3.79 (0 – 8.5)		

a: Healthy controls were significantly younger than people with MS ($p<0.0005$) and their siblings ($p=0.003$), one way ANOVA.
b: Probands with MS were significantly more likely to be female than their unaffected sibling ($p=0.0005$) and healthy controls ($p=0.0006$), Fisher's exact test. There was no significant difference in the gender distribution of the groups between siblings and healthy controls.
c: 3 patients on Avonex, 2 on betaferon, 14 on Rebif, 14 on copaxone, 4 on Natalizumab and 2 on mitoxantrone (last dose >4 months ago for both).

When the ages of participants were divided into 5 year epochs and the age distribution compared between the three groups using an independent samples Kruskal-Wallis test, the significant difference between the groups remained (figure 5.1). It can be seen from figure 5.1 that there was a significant positive skew to the age distribution of the healthy controls, whereas the age distribution

of the participants with MS approached a more Gaussian distribution. Post-hoc testing using a Mann Whitney U Test demonstrated that there was a significant difference between the age distribution between MS probands and healthy controls (p=0.002) and the siblings of people with MS and healthy controls (p=0.001). There was no significant difference in the age group distribution between people with MS and their unaffected siblings (figure 5.1). The gender distribution did not differ significantly between the three groups (Fisher's exact test).

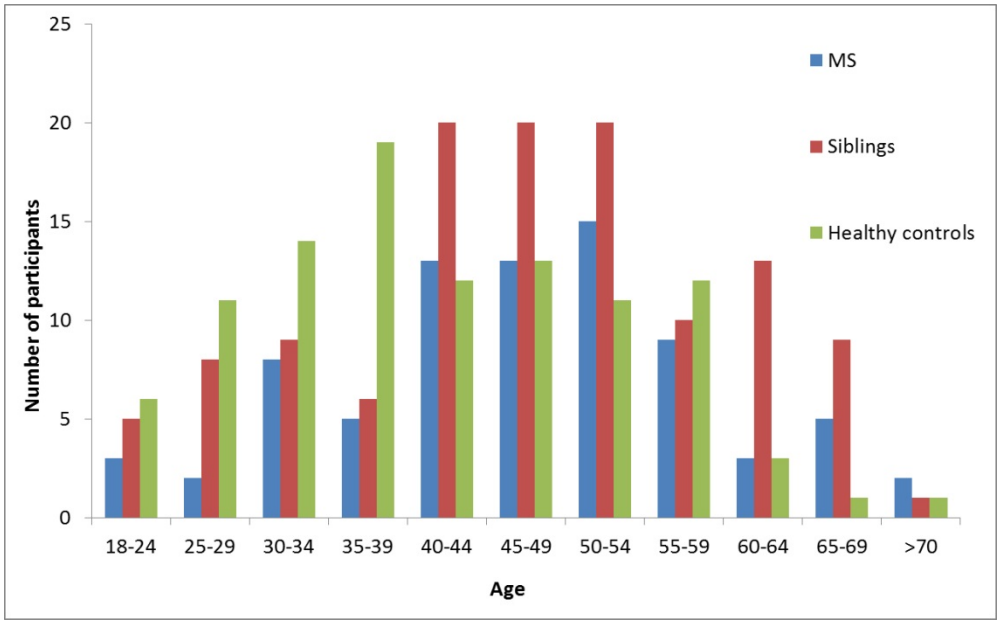


Figure 5.1: Age distribution of MS probands, their unaffected siblings and healthy controls.

Of the 78 people with MS, 45 (69.2%) had relapsing remitting MS, 16 (20.5%) secondary progressive MS and 7 (9.0%) primary progressive MS (table 5.1). 37 (47.4%) participants with MS were on disease modifying treatment. 3 people with MS were taking Avonex, 2 Betaferon, 14 Rebif, 14 Copaxone, 4 Natalizumab and 2 Mitoxantrone. Both patients on Mitoxantrone had received their last dose >4 months ago. No patients had received steroid treatment for their MS within the three months prior to the samples being taken. The EDSS range of the people with MS was 0-8.5, with a mean EDSS of 3.79 (table 5.1). However, the distribution of the EDSS of the people with MS appeared to be bimodal, with the major modal peak at an EDSS of 2, and a minor peak at 6-6.5 (figure 5.2).

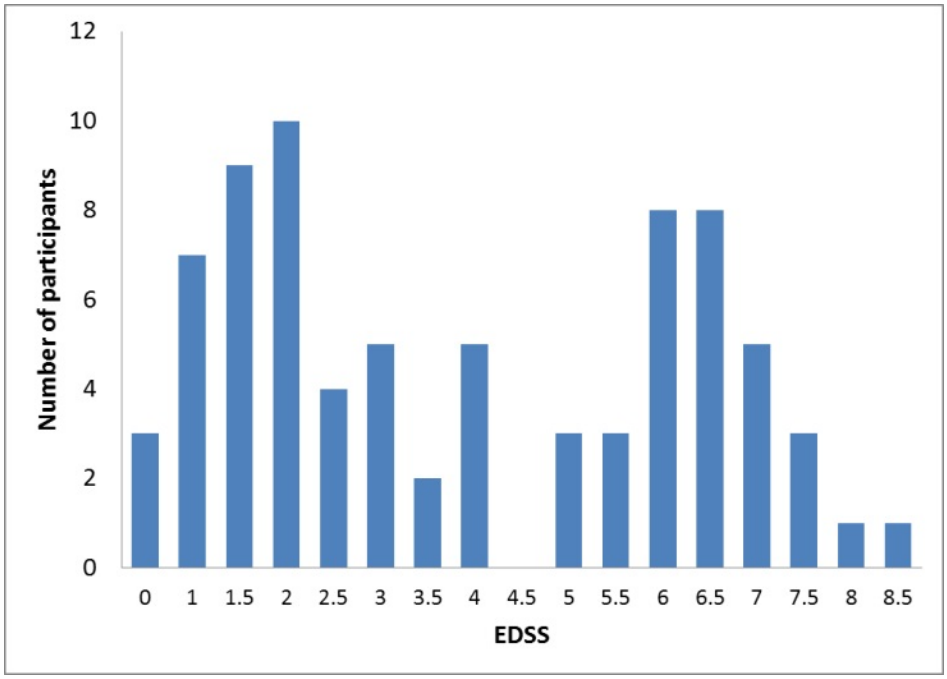


Figure 5.2: Bar chart of EDSS distribution of people with MS, demonstrating the bimodal distribution.

Other demographic feature of the population which have a potential influence on MS risk and were used in the generation of the MS risk score are described under the relevant headings below.

5.1.2. Risk factor distribution

5.1.2.1. Gender

There was a significant difference in the gender distribution between people with MS and both their siblings and healthy controls. People with MS were more significantly more likely to be female than either their unaffected siblings ($p=0.0005$) and healthy controls ($p=0.0006$) (Fisher's exact test) (table 5.1). There was no significant difference between the siblings of people with MS and healthy controls; however as healthy controls had been matched to the sibling group with regard to gender this result was not unexpected.

5.1.2.2. Month of birth

The number of participants enrolled was too low to allow any assessment of difference in month of birth between people with MS, their siblings and healthy controls. The magnitude of the month of birth effect is relatively small (in the region of an odds ratio of 1.05), and so no significant difference would be expected to be seen in a study size of around 100.

However, an attempt was made to examine whether there was a distribution in the season of birth between people with MS, their unaffected siblings, and healthy controls. Using the UK met office definition of the seasons (250), the season of birth of each of the participants was determined from their month of birth (table 5.1). The distribution of season of birth was compared between the three groups using the Chi Square test (4x3 table), and no significant difference was found.

This finding does not mean that the previously published results (128) regarding the significant difference in season of birth distribution between people with MS and healthy controls is not a valid observation – more that the sample size in this population was not sufficient to detect this relatively small effect.

5.1.2.3. Infectious mononucleosis

There was an overall significant difference between the proportion of participants in each group reporting a personal history of infectious mononucleosis (p=0.037, Chi square test) (table 5.2). In post-hoc analysis using a Fisher's exact test, the significant difference was found to lie between the proportion of people with MS (17/78, 21.8%) and healthy controls (10/103, 9.8%) reporting a personal history of infectious mononucleosis (p=0.0345, Fisher's exact test) (table 5.2). There was no significant difference between the proportion of siblings (20/121, 16.5%) reporting a history of infectious mononucleosis when compared to both the MS probands and the healthy control group (Fisher's exact test) (table 5.2).

Table 5.2: Personal history of infectious mononucleosis and IgG titres against EBNA-1

	MS	Siblings	Healthy controls
History of infectious mononucleosis (n; %)	17 (21.8%)	20 (16.5%)	10 (9.8%)[a]
Number with undetectable anti-EBNA-1 IgG (n; %)	3/78 (3.8%)	13/121 (10.7%)	13/103 (12.6%)[b]
Mean anti-EBNA-1 IgG titre of positive samples (IU) (mean; SD)	174.3 (65.2)	136.2 (70.0)	137.3 (63.5)[c]
Number of participants in each quintile (n; % of participants with detectable IgG titres)[c]	Q1: 6 (8.0%)[d, e]	24 (22.2%)	16 (17.8%)
	Q2: 12 (16.0%)	19 (17.6%)	21 (23.3%)
	Q3: 10 (13.3%)	20 (18.5%)	20 (22.2%)
	Q4: 20 (26.6%)	23 (21.3%)	17 (18.9%)
	Q5: 27 (36.0%)	22 (20.4%)	16 (17.8%)
Odds ratio of being assigned to quintile compared to healthy controls (RR; 95% CI)	Q1: 0.40 (0.15-1.09)	1.32 (0.65-2.68)	N/A
	Q2: 0.63 (0.28-1.38)	0.70 (0.35-1.41)	
	Q3: 0.54 (0.23-1.24)	0.80 (0.40-1.59)	
	Q4: 1.56 (0.75-3.26)	1.16 (0.58-2.34)	
	Q5: 2.60 (1.27-5.33)[f]	1.18 (0.58-2.42)	

a: Significant difference between the proportion of MS probands reporting a history of infectious mononucleosis compared to healthy controls (p=0.0345). There was no significant difference between the siblings of people with MS and either their affected siblings or healthy controls.
b: borderline significant difference between the rate of undetectable IgG titres against EBNA-1 between people with MS and healthy controls (p=0.061). No significant difference between people with MS and their unaffected siblings, or between unaffected siblings and healthy controls.
c: quintiles ranging from lowest IgG titre against EBNA-1 (Q1) to highest IgG titre (Q5), defined using combined sibling and healthy control anti-EBNA-1 IgG titres.
d: Significant difference between the quintile distribution of people with MS and their healthy siblings (p=0.0285) and healthy controls (p=0.0164). No significant difference between the distribution between quintiles of the siblings of people with MS and healthy controls.
e: Significant difference between people with MS and their unaffected siblings (p=0.0024) and healthy controls (p=0.0090) when the number in Q1 and Q5 are compared. No significant difference between unaffected siblings and healthy controls.
f: people with MS have a significantly increased odds ratio of being in the highest quintile of IgG titres against EBNA-1 (p=0.01).

The difference in the proportion of people with MS and healthy controls reporting a history of infectious mononucleosis is in keeping with findings from other studies. A recent meta-analysis (262) found that a personal history of infectious mononucleosis was associated with a relative risk of MS of 2.17 (95%CI 1.97-2.39). This increased risk was relatively constant across the different study types examined in the meta-analysis, and the results from the endophenotype cohort would be in keeping with this value – using the differential reported rates of infectious mononucleosis between probands with MS and healthy controls in this study gives a relative risk of MS of 2.24 (95%CI 1.09-4.63) associated with previous infectious mononucleosis.

However, the overall rates of infectious mononucleosis reported in both the MS and sibling groups are significantly higher than expected when compared to data extrapolated from large population-based studies (276). In a study using a large Canadian database, 699/14,362 (4.9%) MS probands reported a history of infectious mononucleosis compared to 165/7,671 (2.2%) spousal controls (276). Using these figures, there is a significantly different rate of infectious mononucleosis between the two MS cohorts, and also when the spousal and sibling cohorts are compared ($p<0.0001$ for both comparisons, Chi square test). The reason for this difference may well be selection bias – large population-based studies include as close as possible to all patients diagnosed with MS in a particular geographical area, minimising as far as possible selection (or volunteer) bias. In contrast, the endophenotype study asked for a relatively small number of volunteers from a relatively large geographical area (the UK). The patient information sheet stated that one area of interest of the study was the role of environmental factors in the development of MS, and many people with MS are aware of the putative link between EBV, infectious mononucleosis and MS. This may well have influenced the nature of the subjects volunteering for this study. It is possible that recall bias played a role in the finding of higher than expected rates of infectious mononucleosis in people with MS and their siblings – however, family members were seen together where possible in an attempt to minimise recall bias. Additionally, recall bias is likely to present in all questionnaire based studies examining the rate of previous infectious mononucleosis in people with MS.

The fact that the rate of infectious mononucleosis in the sibling cohort did not differ significantly from either the cohort with MS or the healthy cohort does not necessarily mean that the rate of infectious mononucleosis in siblings is midway between that of people with MS and healthy controls. Rather, the rate of infectious mononucleosis in siblings may be the same as in people with MS, or healthy controls, or it may actually lie midway between the two, accounting for some of the increased risk seen in the siblings of people with MS. There are no studies examining this; the only work regarding the possible effect of siblings on the rate of infectious mononucleosis in MS found that the rate of self-reported infectious mononucleosis was lower in those MS probands who had a younger sibling born during the first six years of their (the MS probands) life (277). Larger studies are required to explore the rates of infectious mononucleosis in siblings of people with MS, in order to better determine whether there is indeed an increased rate in the sibling population, and, if so, whether this influences the increased MS risk seen in later life.

5.1.2.4. Titre of IgG directed against Epstein Barr virus Nuclear Antigen-1 (EBNA-1)

Serum IgG titres against the Epstein Barr virus Nuclear Antigen-1 (EBNA-1) were measured in all subjects. The methods are described in section 4.2.7.

5.1.2.4.1. Rate of undetectable IgG titres against EBNA-1

3/78 (3.8%) people with MS had undetectable titres of anti-EBNA-1 IgG, compared to 13/121 of their siblings and 13/103 healthy controls (table 5.2). The difference between these proportions did not reach significance (Fisher's exact test), although the difference between the proportion of people with MS and healthy controls with undetectable IgG titres approached significance (p=0.061, Fisher's exact test).

The proportion of people with MS who have undetectable titres of IgG against EBNA-1 has been shown to vary according to the technique used to detect IgG (130). This study was performed using ELISA, which has a lower sensitivity than the accepted gold standard, indirect immunofluorescence (130). However, indirect immunofluorescence is rarely used in practice, as it is relatively labour intensive compared with more modern technologies. Indeed, even the national reference laboratories in the UK have switched to using platform-based technologies, provided by the same manufacturer as the ELISA kit used in this study (Dr Duncan Clark; Consultant Clinical Scientist Royal London Hospital, personal communication). It is therefore possible, if not likely, that a proportion of subjects who are regarded as anti-EBNA-1 IgG negative in this study do in fact have extremely low positive IgG titres against EBNA-1.

5.1.2.4.2. Direct comparison of IgG titres between groups

A comparison of the absolute anti-EBNA-1 IgG titres obtained by using the DiaSorin ELISA was performed. Only those samples with detectable anti-EBNA-1 IgG titres were used in this analysis; those with undetectable IgG titres were excluded.

Although the IgG titres against EBNA-1 for people with MS were found to follow a normal distribution when those samples with undetectable titres were excluded (Shapiro-Wilk test), the distribution of the IgG titres for both the siblings of people with MS and healthy controls deviated significantly from a normal distribution (siblings p=0.001, healthy controls p=0.047, Shapiro-Wilk test). It proved impossible to normalise these distributions, and therefore non-parametric statistical tests were used in the comparison analysis.

There was a significant overall difference in anti-EBNA-1 IgG titres between the three groups (p<0.0005, Kruskal-Wallis test). On post-hoc analysis with a Mann-Whitney U test, there was a significant difference between the anti-EBNA-1 IgG titres between people with MS and their unaffected siblings (p<0.0005, table 5.2 and figure 5.3), and between people with MS and healthy controls (p=0.001, table 5.2 and figure 5.3). There was no difference between the unaffected siblings of people with MS and healthy controls (p=0.971, table 5.2 and figure 5.3).

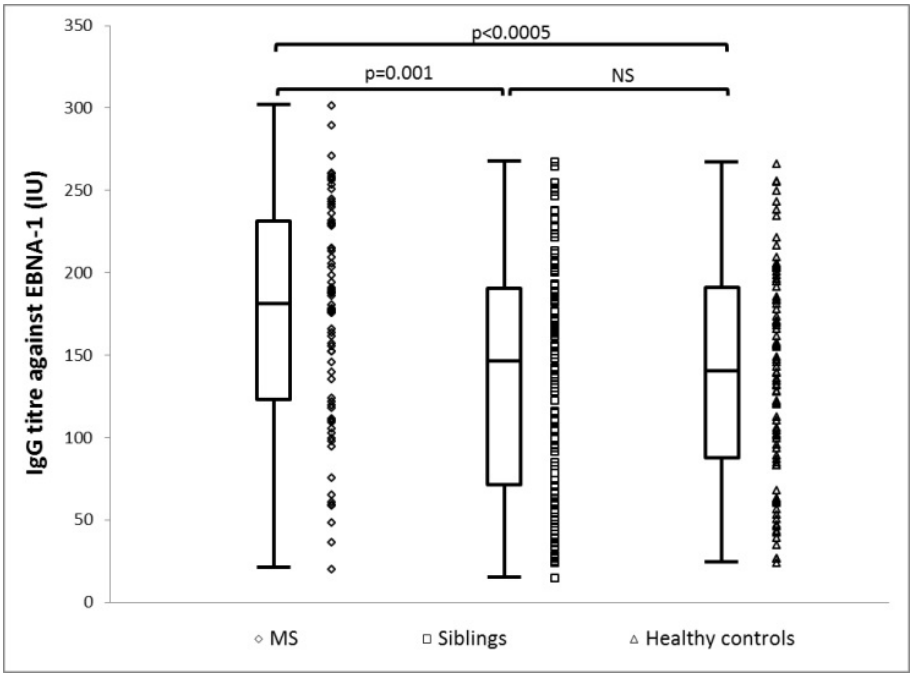


Figure 5.3: Combined box and whisker and scatter plot demonstrating the difference in IgG titres against the EBNA-1 antigen between people with MS, their siblings and healthy controls. The box indicates the interquartile range, bisected by the median, and the whiskers the range.

5.1.2.4.3. Comparison of EBNA-1 quintile allocation between groups

A second analysis was performed, where the proportion of participants in each group with IgG titres against EBNA-1 in each rank quintile was used to compare the groups. This method has previously been used to examine the relationship between anti-EBNA-1 IgG titres and MS (133). The first step in this analysis was to define the quintiles, which was done using the assay and population that the analysis was being performed on, rather than taking values from existing literature. This approach was necessary due to differences between assay results and the lack of a common unit of measurement for anti-EBNA-1 IgG titres (278).

Using the methods described by Levin et al (133), the results for the detectable healthy control and sibling EBNA-1 IgG titres were pooled and organised into rank order. These were then used to define the cut-off values for the five rank order quintiles. Using these cut-off values, each participant in the study was then allocated to the appropriate quintile according to their anti-EBNA-1 IgG titre. Quintile 1 represents the lowest detectable IgG titres against EBNA-1, and quintile 5 the highest. The number of participants in each group assigned to each quintile were then compared using Chi square test on a 3x5 table. Further post-hoc analysis was performed to compare pairs of groups using Chi square (2x5 table). The number of participants in the extreme quintiles (Q1 and Q5) were compared between the three groups using Chi square (3x2 table) with post-hoc analysis between pairs of groups using Fisher's exact test. Additionally, the odds ratio of both people with MS and healthy controls being assigned to each quintile was determined using standard methods.

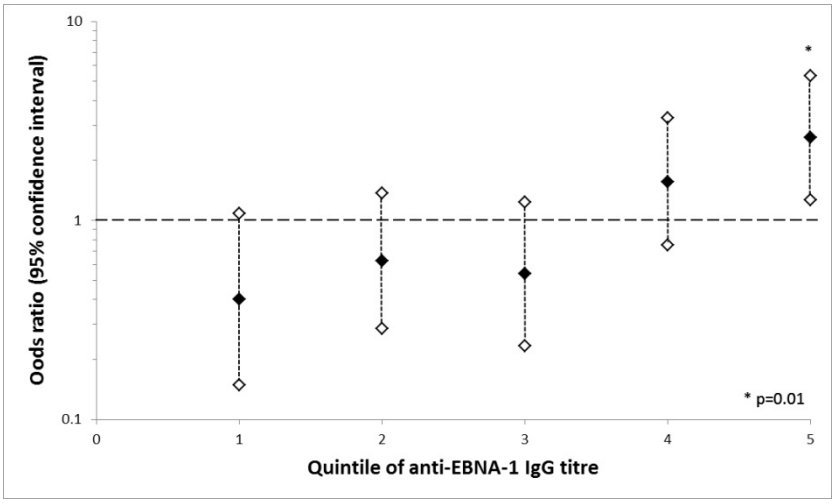
The number of participants in each group assigned to each quintile is given in table 5.2. There was an overall significant difference between the three groups when the distribution of participants across all five quintiles was compared (p=0.0388, Chi square). This significance was maintained when the quintile distribution of people with MS was compared to the distribution of their healthy siblings (p=0.0285, Chi square) and healthy controls (p=0.0164, Chi square) (table 5.2). There was no

significant difference between the distribution of the siblings of people with MS and healthy controls (table 5.2).

When comparing the proportion of participants in the extreme quintile categories (quintiles 1 and 5), there was a highly significant difference between the three groups overall (p=0.0052, Chi square). This was maintained on post-hoc testing when people with MS were compared to their unaffected siblings (p=0.0024, Fisher's exact test) and healthy controls (p=0.0090, Fisher's exact test). There was no significant difference when the unaffected siblings were compared to healthy controls (table 5.2).

Finally, the odds ratio of being assigned to a given quintile was compared between both people with MS and their unaffected siblings and healthy controls (table 5.2 and figures 5.4a and b). People with MS had a significantly increased odds ratio of being in the highest quintile of anti-EBNA-1 IgG titres when compared to healthy controls (p=0.01) (figure 5.4a). However, the odds ratio of people with MS being in the lowest quintile of IgG titres against EBNA-1 did not differ significantly from healthy controls. The unaffected siblings of people with MS did not have an odds ratio deviating significantly from 1 with respect to being in any of the five quintiles of IgG titres against EBNA-1 when compared to healthy controls (figure 5.4b).

The finding of increased IgG titres against EBNA-1 is in keeping with that previously published (132, 133, 279). This finding does not appear to extend to the unaffected siblings of people with MS. The overall distribution of anti-EBNA-IgG titres in the sibling group appears similar to that of healthy controls, and there is no significant difference between healthy controls and the siblings of people with MS with respect to the relative risk of being assigned to any of the quintiles of anti-EBNA-1 IgG titre.



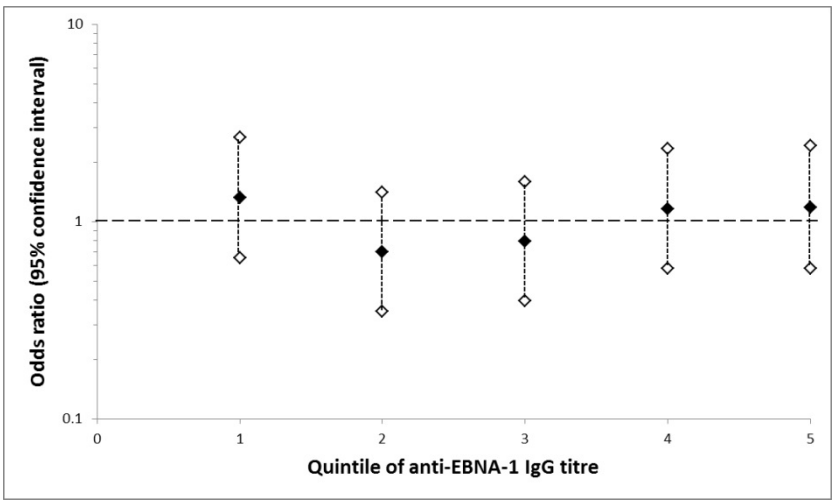


Figure 5.4: (a) OR of people with MS being in a given quintile of IgG titres against the EBNA-1 antigen. The quintiles were defined using the combined values for the unaffected siblings of people with MS and healthy controls. The solid diamond represents the calculated OR, and the line between the two unfilled diamonds the 95% confidence interval. **(b)** OR of the unaffected siblings of people with MS being in a given quintile of IgG titres against the EBNA-1 antigen. The quintiles were defined using the combined values for the unaffected siblings of people with MS and healthy controls. The solid diamond represents the calculated OR, and the line between the two unfilled diamonds the 95% confidence interval.

5.1.2.5. Smoking status

The reported rates of "ever smoking" in each group are given in table 5.3. As described in section 4.2.8, a cotinine cut-off of 3.08ng/ml was used to define biochemical current smokers (216). Although more participants in each of the three groups had cotinine levels meeting the definition of "biochemical current smoking" than the number who reported current smoking, the proportion of participants classified as "current smokers" did not differ significantly based on the method (self-report vs. biochemical classification) used to define "current smoking". All of those participants who did not report a history of "current smoking" but who were classified as "biochemical smokers" reported a history of previous smoking. For the purposes of this study, those participants who had a serum cotinine level >3.08ng/ml were classified as "current smokers" in terms of the risk score generation.

Table 5.3: Number of participants giving a history of ever- or current smoking, and cotinine status of participants.

	MS (n=78)	Siblings (n=121)	Healthy controls (n=103)
Ever smoking (reported; n, %)	41 (52.6%)	54 (44.6%)	28 (27.2%)
Current smoking (reported; n, %)	13 (16.7%)	14 (11.6%)	8 (7.8%)
Biochemical smoking (serum cotinine >3.08 ng/ml)	16 (20.5%)	18 (14.9%)	12 (11.7%)

The distribution of serum cotinine levels overall followed a bimodal distribution (figure 5.5). There was a clear distinction between "biochemical smokers" and "non-smokers", with no participants having a cotinine level within ±1ng/ml of the cut-off level. Those participants who had a cotinine level ±2ng/ml within the cut-off level (i.e. 1.08–5.08ng/ml) had their serum samples re-assayed as described in the methods section. All of those participants who had samples re-assayed demonstrated consistency between being either a "biochemical smoker" or "non-smoker" with respect to all four results.

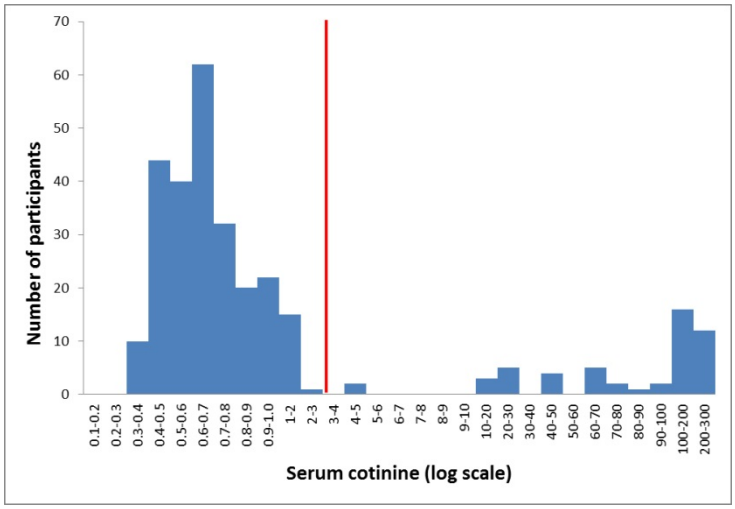


Figure 5.5: Distribution of serum cotinine levels of all participants in the study. The red line indicates the cut-off point for defining biochemical current smoking.

There was an overall significant difference in the proportion of participants reporting a history of "ever smoking" in the three groups (p=0.0014, Chi square 2x3 table). Post-hoc analysis using Fisher's exact test revealed that significant differences in the proportion of participants reporting a smoking history lay between people with both MS and healthy controls (p=0.0007), and the unaffected siblings of people with MS and healthy controls (p=0.0082). There was no significant difference in the proportion of people with MS and their unaffected siblings reporting a history of "ever smoking".

When a similar analysis was performed with the proportion of participants reporting a history of "current smoking", there was no overall significant difference between the three groups (Chi square 2x3 table). Post-hoc analysis between pairs of groups revealed no significant differences.

Finally, an analysis was performed using the proportion of subjects in each group who were classified as "biochemical current smokers", i.e. those with a serum cotinine level >3.08ng/ml. Similarly to the results obtained with self-reported smoking, there was no significant difference between the three groups (Chi square 2x3 table).

The finding of increased rates of "ever smoking" associated with MS compared to healthy controls could therefore be seen in the participants in this study. The fact that unaffected siblings of people with MS have similar rates of smoking to people with MS is not necessarily a surprise – there is evidence that factors within individual cohorts of families influence smoking rates (280). When

demographic and socioeconomic factors are controlled for, there is evidence that collective familial norms regarding attitudes to smoking are the strongest predictor of smoking status (280). It is therefore likely that the siblings of the healthy control cohort would display smoking behaviours similar to the healthy controls studied. Unfortunately, in the context of this study, such behavioural transmission is almost impossible to control for in a practical sense. It is therefore difficult to come to a definite conclusion regarding the effect of smoking on a sibling risk of MS, although it seems likely that this may well influence risk to some degree.

5.1.2.6. Serum 25-hydroxyvitamin D levels

Serum 25-hydroxyvitamin D (25-OHvD) levels were measured and the raw values deseasonalised as described in section 4.2.9. The deseasonalised values were used in all analysis.

5.1.2.6.1. Serum levels of 25-hydroxyvitamin D

The raw serum 25-OHvD levels were initially tested for normality using the Shapiro-Wilk test. Although the distribution of the serum 25-OHvD levels in people with MS did not differ significantly from a normal distribution ($p=0.06$), the distributions in both the sibling and healthy control groups were significantly different from normal ($p<0.0005$ for both). Although a natural logarithmic transformation was able to successfully normalise the distributions of serum 25-OHvD for both the sibling ($p=0.222$) and healthy control ($p=0.357$) groups, it resulted in the serum 25-OHvD results for the MS group significantly deviating from a normal distribution ($p<0.0005$). Non-parametric statistical tests were therefore used in the analysis of the raw serum 25-OHvD values.

When the three groups were compared using a Kruskal-Wallis test, there was a non-significant difference between the three groups overall ($p=0.093$). Similarly, when the medians of the three groups were compared there was no significant difference between the three groups ($p=0.173$). However, when pairwise comparisons between the serum 25-OHvD levels were performed, people with MS had significantly higher serum 25-OHvD levels than healthy controls ($p=0.048$, Mann Whitney U test) (table 5.4 and figure 5.6). There was a trend towards a significant difference between the people with MS and their unaffected siblings ($p=0.061$) (table 5.4 and figure 5.6). There was no significant difference in serum 25-OHvD levels between the unaffected siblings of people with MS and healthy controls ($p=0.739$).

Table 5.4: Distribution of serum 25-hydroxyvitamin D levels

	MS (n=78)	Siblings (n=121)	Healthy controls (n=103)
Deseasonalised serum 25-OHvD level (nmol/l; median; SD)	66.50 (31.04)[b]	61.83 (21.53)	58.64 (25.05)
Number of subjects in each quintile of serum 25-OHvD level[a] (n;%)	Q1: 34 (43.6%) Q2: 13 (16.7%) Q3: 8 (10.3%) Q4: 9 (11.5%) Q5: 14 (17.9%)[c]	62 (51.2%) 25 (20.7%) 20 (16.5%) 8 (6.6%) 6 (5.0%)	58 (56.3%) 17 (16.5%) 9 (8.7%) 10 (9.7%) 9 (8.7%)
Odds ratio of being assigned to quintile compared to healthy controls (OR; 95% CI)	Q1: 0.60 (0.33-1.09) Q2: 1.01 (0.46-2.23) Q3: 1.19 (0.44-3.25) Q4: 1.21 (0.47-3.15) Q5: 2.28 (0.93-5.59)	0.82 (0.48-1.38) 1.32 (0.67-2.60) 2.07 (0.90-4.77) 0.66 (0.25-1.74) 0.48 (0.19-1.59)	N/A
Odds ratio of being assigned to quintile compared to siblings (OR; 95% CI)	Q1: 0.74 (0.41-1.30) Q2: 0.77 (0.37-1.61) Q3: 0.66 (0.28-1.53) Q4: 1.84 (0.68-5.00) Q5: 4.19 (1.54-11.44)[d]	N/A	
Number reporting regular (>3x/wk) vitamin D supplementation	15 (19.2%)[e]	4 (3.3%)	5 (4.9%)

a: cut-off values for quintiles as defined by Munger et al (147):

Q1: <63.2 nmol/l
Q2: 63.3-75.3 nmol/l
Q3: 75.4-84.8 nmol/l
Q4: 84.9-99.1 nmol/l
Q5: >99.2 nmol/l

b: MS significantly higher than healthy controls; p=0.048. Trend towards MS being higher than unaffected siblings, p=0.061

c: Significant difference between quintile distribution between people with MS and unaffected siblings; p=0.0205

d: Significantly increased OR of people with MS being in highest quintile than siblings, p=0.005

e: Significantly higher proportion of people with MS regularly supplementing with vitamin D compared to both unaffected siblings (p=0.0003) and healthy controls (p=0.0033).

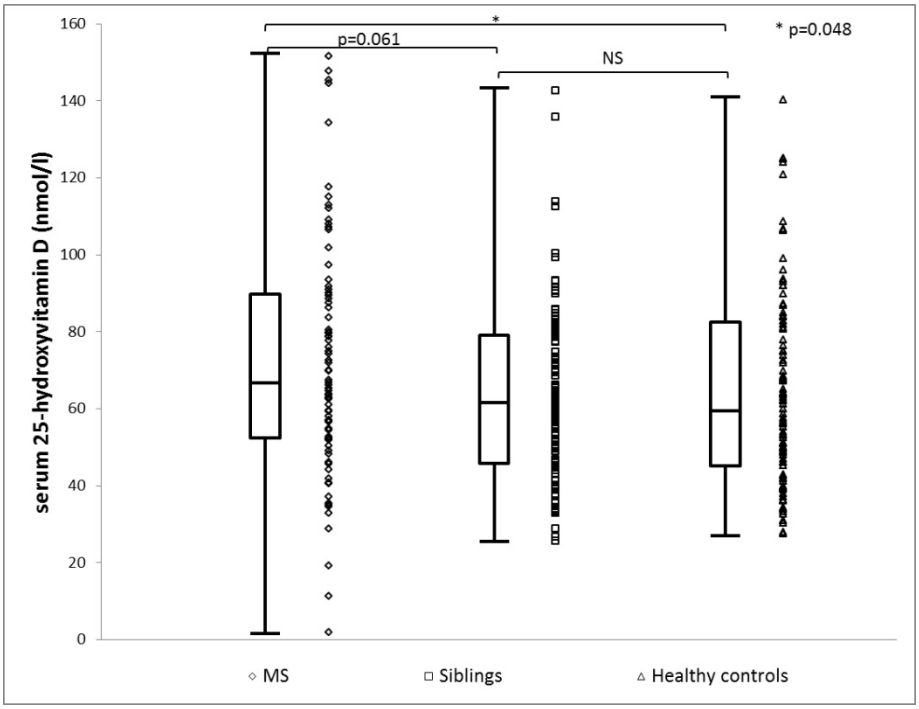


Figure 5.6: Combined box and whisker and scatter plot demonstrating the difference in serum 25-OHvD levels between people with MS, their siblings and healthy controls. The box indicates the interquartile range, bisected by the median, and the whiskers the range.

5.1.2.6.2. Quintile analysis of serum 25-hydroxyvitamin D levels

As previously described in section 4.2.9, the serum values 25-OHvD for each individual were matched to the relevant quintile described by Munger et al (147), and that quintile assigned to the individual concerned. The serum levels of 25-OHvD used to define each of the quintiles are given in the footnote to table 5.4. The distribution of the quintiles across the three groups was then compared.

An initial comparison across the three groups was performed using a Chi square test on a 3x5 table. There was a trend towards a significant difference between the groups when the three groups were compared (p=0.0588, Chi square) (table 5.4 and figure 5.7). When pairwise comparisons were made, there was a significant difference between the 25-OHvD distribution across quintiles between people with MS and their unaffected siblings (p=0.0205, Chi square 2x5 table). However there was

no difference in the quintile distribution between people with MS and healthy controls (p=0.3274, Chi square 2x5 table). Similarly, there was no significant difference in the quintile distribution between the unaffected siblings of people with MS and healthy controls (p=0.2635).

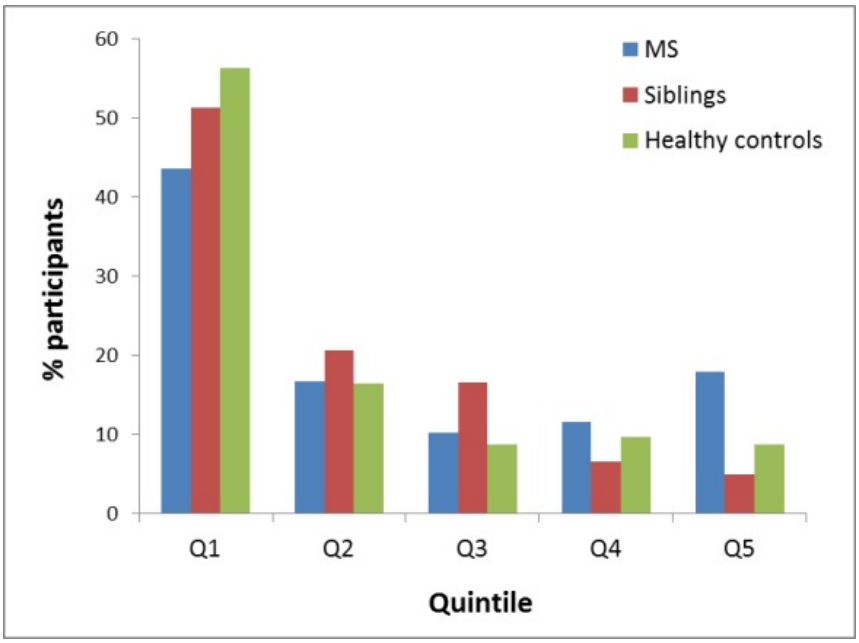


Figure 5.7: The proportion of subjects in either the MS group, the unaffected siblings or healthy controls who were allocated to each of the five pre-defined quintiles of serum 25-OHvD levels. The quintiles were defined using the values given by Munger et al (147).

When the number of participants in the extreme quintiles (Q1 and Q5) were compared between the three groups there was an overall significant difference (p=0.0101, Chi square 3x2 table). When post-hoc pairwise analysis was performed using Fisher's exact test, there was a highly significant difference between the number of participants in Q1 and Q5 between people with MS and their unaffected siblings (p=0.0059, Fisher's exact test). There was a trend towards a difference between people with MS and healthy controls (p=0.0573, Fisher's exact test), and no difference between the unaffected siblings of people with MS and healthy controls (p=0.4255) (table 5.4).

When the odds ratio of being in a given quintile of serum 25-OHvD levels was calculated for people with MS and their unaffected siblings compared to healthy controls, there was no significant

increase or decrease in the chance of being in any quintile in either group (data not shown). However, the odds ratio of people with MS being in the highest quintile of serum 25-OHvD levels was significantly increased when they were compared to their unaffected siblings (p=0.005) (figure 5.8).

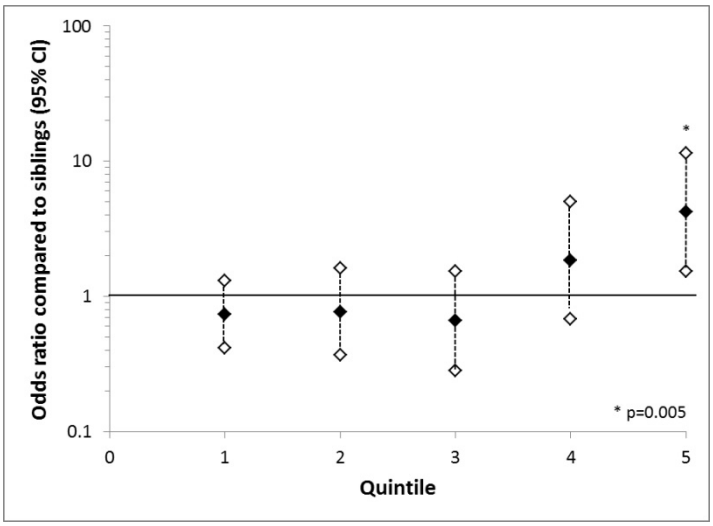


Figure 5.8: Odds ratio (OR) of being in a given pre-defined quintile of serum 25-hydroxyvitamin D for participants with MS compared to their unaffected siblings. The solid diamond represents the calculated OR, and the line between the two unfilled diamonds the 95% confidence interval.

5.1.2.6.3. Effect of vitamin D supplementation

There was an unexpected significant increase in the proportion of people with MS being in the highest quintile of serum 25-OHvD. This finding was in contrast to multiple previous publications associating MS with a low serum 25-OHvD (281). It seems plausible that this increase was due to post-diagnosis behavioural modification in the MS group, with people with MS being more likely to take vitamin D supplements regularly, especially given the increasing awareness of the prevalence and potential effects of vitamin D deficiency.

15/78 people with MS reported regularly taking vitamin D supplementation (>3x/week), compared to 4/121 of their siblings and 5/103 healthy controls (table 5.4). This difference in the proportion

supplementing between the groups was highly statistically significant ($p<0.0001$, Chi square 3x2 table). On post-hoc pairwise analysis there was a significant difference in the proportion of people with MS and their unaffected siblings regularly supplementing ($p=0.0003$, Fisher's exact test) and the proportion of people with MS and healthy controls regularly supplementing ($p=0.0033$, Fisher's exact test) (table 5.4). There was no significant difference between the proportion of unaffected siblings and healthy controls taking regular vitamin D supplementation ($p=0.7357$) (table 5.4).

However, those people with MS taking regular vitamin D supplementation did not account for all of those people with MS in the highest quintile of serum vitamin D. Only 4 of the 15 people with MS who were regularly supplementing with vitamin D were in the highest quintile (accounting for 4/14 of those with MS in the highest quintile); the other supplementing participants were equally spread between quintiles 1, 2 and 3. The reason for the bulge of people with MS in the highest quintile of serum vitamin D levels therefore remains unexplained to some degree. Given the fact that post-diagnosis behavioural modification therefore cannot be assumed to account for the observed bulge in the highest quintile of serum vitamin D, the samples giving these unexpected results were not removed from the cohort when calculating the overall MS risk score.

5.1.2.6.4. Historical serum 25-hydroxyvitamin D levels in people with MS

To further test the hypothesis that the increased proportion of patients with MS with serum 25-OHvD levels in the highest quintile was a result of behavioural modification, those subjects with MS who had previously had serum 25-OHvD levels measured at the Royal London Hospital were identified. There were 10 subjects who had serum 25-OHvD levels recorded prior to 2011. Deseasonalised values were used in this comparison. Results for each participant are given in table 4.15. 8/10 patients had an increase in serum 25-OHvD following the initial measurement of the serum level pre-2011. When the pre-2011 and endophenotype study vitamin D levels were compared, there was a significant difference between the two groups ($p=0.05$, paired t-test), despite the relatively small number of participants who had both values available (table 5.5 and figure 5.9).

Exclusion of the single outlier with an unusually high serum 25-OHvD level pre-2011 (MS-070) did not lead to a change in the significant p-value of 0.05.

Table 5.5: Comparison of raw pre-2011 and endophenotype study serum 25-hydroxyvitamin D levels

Subject	Pre-2011 serum 25-OHvD level (nmol/l)	Endophenotype serum 25-OHvD level (nmol/l)
MS-007	35	49
MS-011	30	36
MS-013	60	66
MS-027	49	13
MS-037	43	121
MS-042	69	71
MS-052	74	94
MS-067	29	107
MS-070	153	151
MS-080	51	80
Mean (SD)	**59.3 (36.4)[a]**	**78.8 (41.2)**

a: p=0.05 for difference between pre-2011 25-OHvD levels and those measured as part of the endophenotype study (paired t-test).

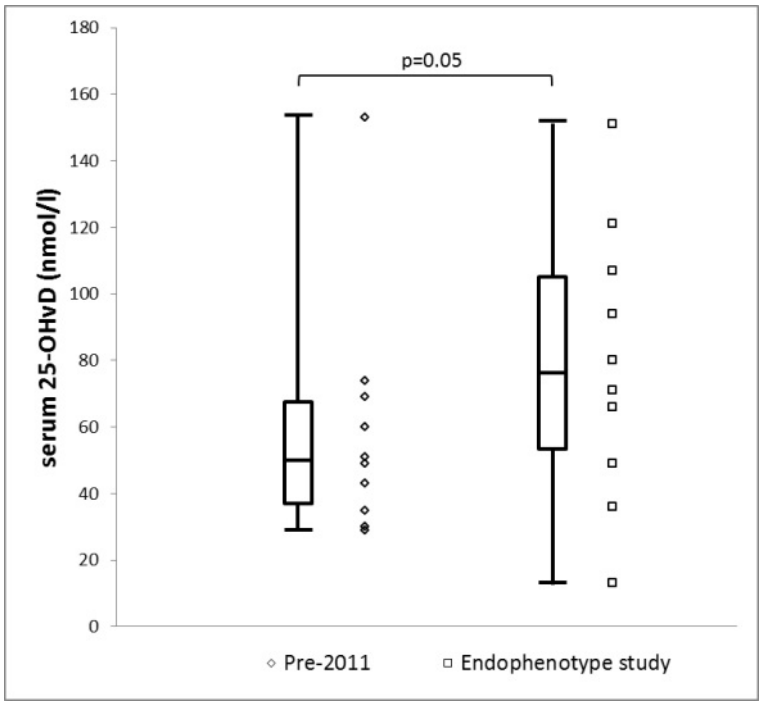


Figure 5.9: Comparison between pre-2011 serum 25-hydroxyvitamin D levels and levels measured as part of the endophenotype study. The box indicates the interquartile range, bisected by the median, and the whiskers the range.

An inherent weakness of this study is the effect of post-diagnosis behavioural modification in both the MS and unaffected sibling groups. However, the evidence that this post-diagnosis behavioural effect is solely responsible for the relatively high vitamin D levels in this study is weak. Not all of those supplementing have very high vitamin D levels, and not all of those with high vitamin D levels report regularly supplementing. There is little evidence to support a theory that a diagnosis of MS has affected the behaviour of unaffected siblings with respect to vitamin D supplementation – supplementation rates do not differ significantly from healthy controls in this cohort, and serum 25-OHvD levels are similar between unaffected siblings of people with MS and healthy controls.

5.1.2.7. HLA-DRB1*1501 haplotype

Of the 64 people with MS on whom full genetic information was available, 4 were heterozygote for the HLA-DRB1*1501 allele, 33 were homozygotes (i.e. had a single copy of the allele), and 27 did not carry the HLA-DRB1*1501 allele. Of the 92 siblings on whom full genetic information was available, 4 were heterozygous for the allele, 40 homozygous, and 48 did not carry the allele. Of the 99 healthy controls who had full genetic data available, 2 were homozygous and 18 were heterozygous for the HLA-DRB1*1501 allele, and 79 did not carry the allele (table 5.6).

Table 5.6: Rates of carriage of HLA-DRB1*1501

	Homozygote for HLA-DRB1*1501 (n;%)	Heterozygote for HLA-DRB1*1501 (n;%)	No carriage of HLA-DRB1*1501 (n;%)
MS (n=64)	4 (6.3%)	33 (51.6%)	27 (42.2%)[a]
Sibling (n=92)	4 (4.3%)	40 (43.5%)	48 (52.2%)
Healthy control (n=99)	2 (2.0%)	18 (18.2%)	79 (79.8%)

a: significant difference in the rate of HLA-DRB1*1501 carriage between healthy controls and people with MS (p=0.0001) and the siblings of people with MS (p=0.0001).

There was a highly significant difference overall regarding the rate of HLA-DRB1*1501 carriage ($p<0.0001$, Chi square 3x2 table). There was a non-significant difference between people with MS and their unaffected siblings with respect to HLA-DRB1*1501 carriage. There was a highly significant difference between healthy controls and both people with MS (p=0.0001, Fisher's exact test) and their unaffected siblings (p=0.0001, Fisher's exact test) regarding the rate of carriage of the HLA-DRB1*1501 allele (table 5.6).

5.1.2.8. MS risk SNPs identified by GWAS

For the 61 MS risk SNPs (i.e. all risk alleles apart from the possession of HLA-DRB1*1501) identified by the 2011 GWAS (103), observed frequencies in the background population were taken from the immunobase website. It was then possible to compare observed and expected frequencies of these SNPs in the three populations studied. In the MS population, 9 SNPs were seen at significantly different frequency than would be expected. In the sibling population 5 SNPs were seen at significantly different frequency than expected, and 5 were seen at significantly different frequency from expected in the healthy control population. However, when a Bonferroni correction for multiple testing was applied, none of these differences remained significant, and thus this data is not presented.

In addition, the GWAS was performed using a population of European ancestry from a number of countries including Finland, Sweden, Norway, Denmark, Poland, Spain and Italy. Background expected genotype frequencies were not available divided by country of origin, and it may well be that the frequencies of some variants differ considerably between, for example, Spain and the UK.

Clearly with a relatively small sample size, differences in the observed:expected ratios would not necessarily be expected to be demonstrated in all SNPs that have been associated with a minor alteration in MS risk, and given the concern regarding the non-specificity of the population studied in the GWAS and the non-significant p-values this was not taken any further.

5.2. MS Risk score

5.2.1. Introduction

Having generated the putative MS risk score using the methods described by de Jager et al (157), it was important to examine not only the values and distribution of the risk score between the three groups, but also to examine its robustness. In addition, it was necessary to examine the effect of the post-diagnosis behavioural modification regarding vitamin D supplementation on the overall MS risk score. In order to provide an indication of the relative effect of the genetic contribution outside of HLA-DRB1*1501 two risk scores were generated, one using HLA-DRB1*1501 only, and a second score using the full genetic information elicited from the immunochip results. Robustness analysis, where serum 25-OHvD levels were excluded from the MS risk score generation was performed, and the effects of this exclusion were analysed. In addition, given the uncertainty regarding the inclusion of serum 25-OHvD levels into the model, various post-hoc analyses were performed on the sibling group, examining the effects on an individual's score of excluding this variable.

A receiver operating characteristic (ROC) curve was created for MS vs. healthy controls for each of the risk scores generated. In a ROC curve true positives (sensitivity) are plotted against false positives (1-sensitivity) for all possible score values. The area under a ROC curve (AUC) provides a single figure that acts as an estimate of the overall expected performance of the score. A score that is randomly distributed would give an AUC of 0.5. A score that discriminates perfectly between MS and HC would give an AUC of 1.0. The magnitude of an AUC between 0.5 and 1.0 provides a measure of the difference in scores between the two groups (MS and HC). The confidence interval is the range in which you can be 95% certain that the true AUC resides.

In order to assess the potential clinical utility of the putative MS risk score, the overall risk score distribution was partitioned using the healthy control risk score distribution, and the risk of MS for subjects in each partitioned category assessed. This provided further information regarding the

potential predictive value of the MS risk score, and also enabled an assessment of the validity of the score. Finally, trends across the risk score regarding the odds ratio of having MS were examined, in order to test both the distribution of risk and also to perform a further validity assessment.

These multiple analyses enabled a thorough examination of the putative MS risk score generated, which in turn provides a wealth of data surrounding both further strategies for ensuring validity of the score, and hints at the potential clinical utility.

5.2.2. Including genetic contribution from HLA-DRB1*1501 only

The initial MS risk score was generated using the genetic information regarding carriage of HLA-DRB1*1501 only. The distribution of the MS risk score was Gaussian for people with MS, unaffected siblings and healthy controls; therefore parametric statistical tests were used to compare the groups. Details regarding the MS risk scores obtained by each group are given in table 5.7. There was a significant difference in the MS risk scores between the three groups ($p<0.0005$; one-way ANOVA) (table 5.7 and figure 5.10). Post-hoc testing using a Bonferroni correction revealed significant differences between all three groups (table 5.7 and figure 5.10). People with MS had a significantly higher MS risk score than both their unaffected siblings ($p<0.0005$) and healthy controls ($p<0.0005$). There was also a significant difference between mean MS risk scores of the unaffected siblings of people with MS and the healthy controls ($p=0.042$) (table 5.7 and figure 5.10).

A receiver operating characteristic (ROC) curve comparing people with MS with healthy controls (i.e. excluding unaffected siblings) generated an area under the curve of 0.772 (95% CI 0.702 – 0.842) (table 5.8 and figure 5.11).

Table 5.7: Risk scores for each group

	MS (n=73)	Siblings (n=107)	Healthy controls (n=99)
Risk score including genetic contribution from HLA-DRB15*1501 only (mean; SD)	2.82 (1.18)[a]	1.98 (1.38)[b]	1.53 (1.34)
Risk score including genetic contribution from all MS risk alleles (mean; SD)	9.71 (1.38)[c]	8.83 (1.47)	8.00 (1.49)
Risk score including genetic contribution from HLA-DRB1*1501 only; excluding serum 25-OHvD level (mean; SD)	3.13 (1.12)	2.16 (1.33)	1.74 (1.32)
Risk score including genetic contribution from all MS risk alleles; excluding serum 25-OHvD level (mean; SD)	10.02 (1.38)	9.00 (1.44)	8.20 (1.48)

a: $p<0.0005$ for difference between MS and siblings and MS and HC
b: $p=0.042$ for difference between siblings and HC
c: $p<0.0005$ for difference between MS and siblings, MS and HC and siblings and HC

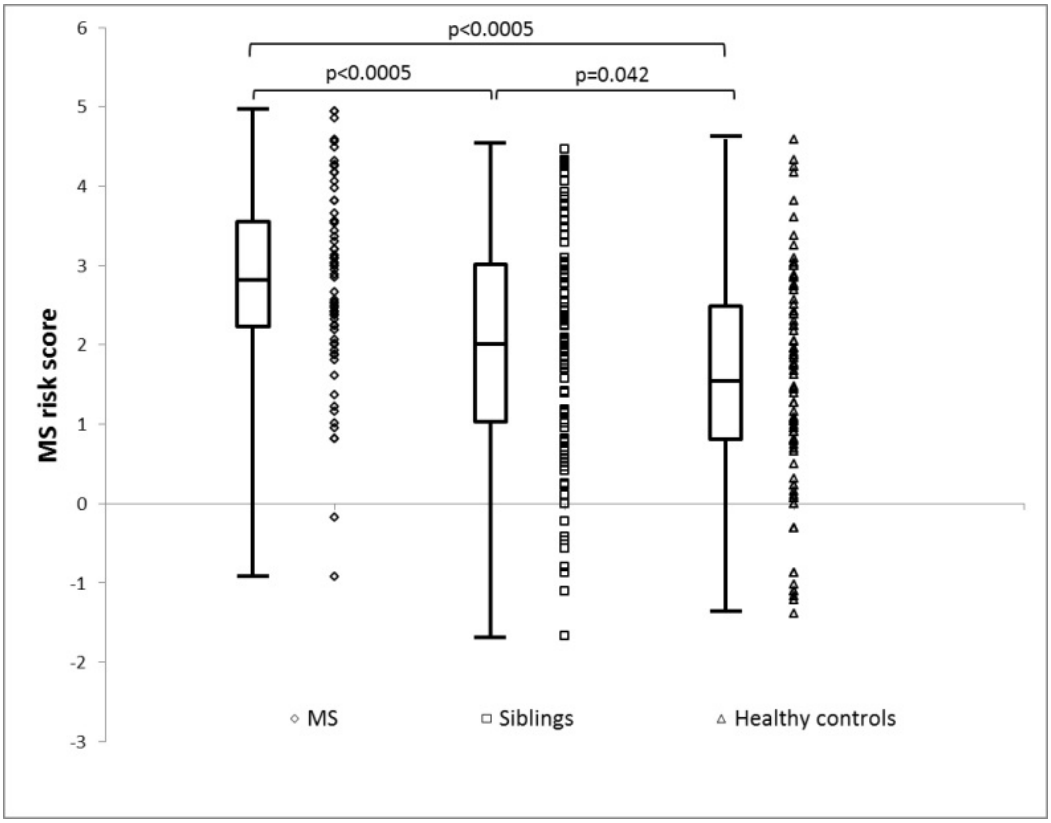


Figure 5.10: Combined scatter and box-and-whisker plot demonstrating the MS risk score distribution between people with MS, their unaffected siblings and healthy controls where HLA-DRB1*1501 is the only genetic information used to derive the risk score. The box indicates the interquartile range, bisected by the median, and the whiskers the range.

Table 5.8: Receiver operating curve (ROC curve) characteristics for the MS risk scores generated.

	Area under curve
Risk score including genetic contribution from HLA-DRB15*1501 only (AUC; 95% CI)	0.772 (0.702 – 0.842)
Risk score including genetic contribution from all MS risk alleles (AUC; 95% CI)	0.801 (0.735 – 0.866)
Risk score including genetic contribution from HLA-DRB1*1501 only; excluding serum 25-OHvD level (AUC; 95% CI)	0.800 (0.733 – 0.868)
Risk score including genetic contribution from all MS risk alleles; excluding serum 25-OHvD level (AUC; 95% CI)	0.818 (0.754 – 0.881)

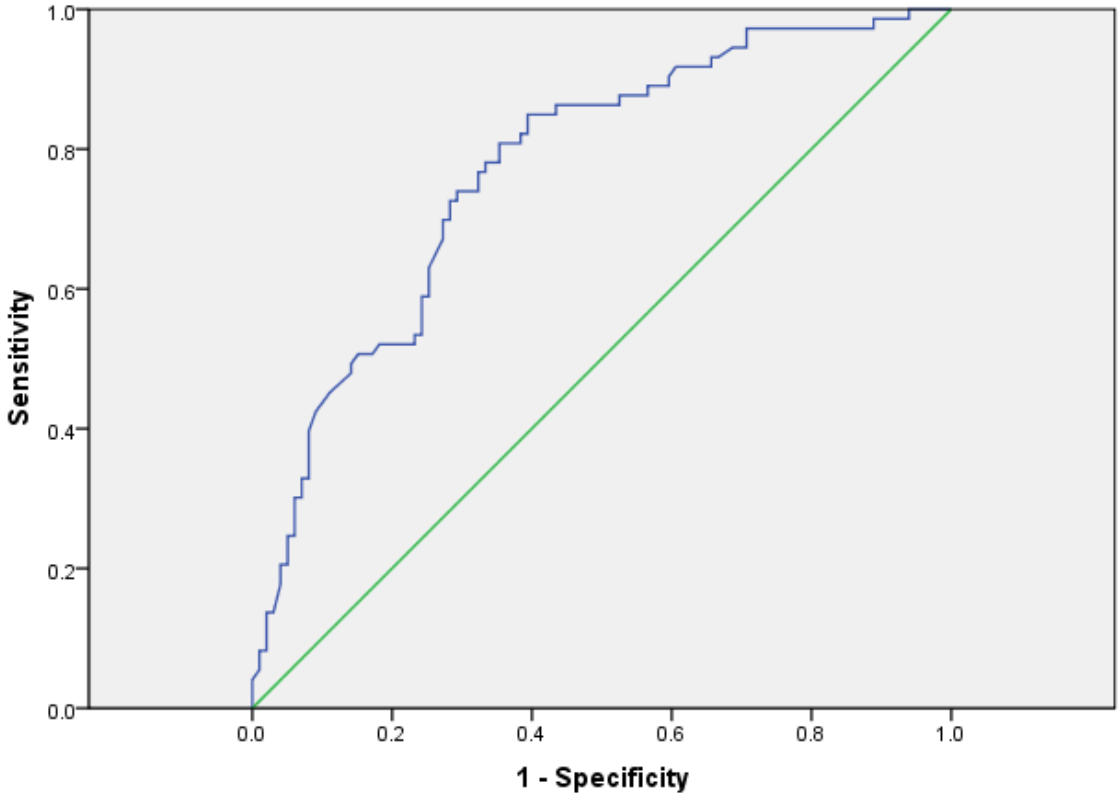


Figure 5.11: A receiver operating characteristic (ROC) curve generated by the MS risk score including HLA-DRB1*1501 only when the score of people with MS is compared to that of healthy controls.

Finally, seven categories of MS risk score were defined using the risk scores of the healthy control population. These were defined using the mean and standard deviation of the healthy control risk score, according to the methods described by de Jager et al (157). The seven categories were defined as +/- 0·25, 0·75, and 1·25 standard deviations from the mean; the extreme categories (categories 1 and 7) were less than 1·25 or greater than 1·25 SD from the mean respectively, with category 1 representing the lowest risk score, and category 7 the highest. By partitioning the risk scores in this way, a direct comparison of the proportion of participants obtaining extreme risk scores between groups was possible. An initial calculation compared the proportion of people with MS or siblings in a given category to the proportion of healthy controls in that category (table 5.9 and figure 5.12).

Table 5.9: Number of participants and odds ratio for being assigned to the categories defined using the healthy control MS risk score results. The proportion of healthy control subjects in the MS risk score category is used as the reference, and the proportion of people with MS or their unaffected siblings in this risk score category compared to the reference.

Category	MS (n)	MS (OR; 95% CI)	Siblings (n)	Siblings (OR; 95% CI)	Healthy control (n)
1	2	0.2254 (0.0484-1.0498)	8	0.6465 (0.2488-1.6798)	11
2	0	N/A (0 patients)	10	0.9175 (0.3647-2.3082)	10
3	4	0.3246 (0.1030-1.0232)	10	0.5773 (0.2463-1.3532)	15
4	5	0.2298 (0.0830-0.6359)	16	0.5495 (0.2722-1.1093)	24
5	9	1.2516 (0.4811-3.2560)	12	1.1242 (0.4628-2.7311)	10
6	27	2.1801 (1.1081-4.2892)[a]	32	1.5848 (0.8397-2.9910)	21
7	26	6.29 (2.6439-14.9764)[b]	19	2.4560 (1.0223-5.9003)[c]	8

a: p=0.02; b: p<0.0001; c: p=0.04

There was a significant increase in the odds ratio of people with MS being in the two highest risk score categories when compared to healthy controls (OR 2.18 and 6.29, p=0.02 and p<0.0001 for highest two categories respectively) (table 5.9 and figure 5.12a). There was also a significant increase in the odds ratio of the unaffected siblings of people with MS being in the highest risk score category when compared to healthy controls (OR 2.46, p=0.04) (table 5.9 and figure 5.12b). A regression model was used to assess the trend across the groups; the OR was assumed to be a continuous variable for the purposes of this model. Models were created for linear, logarithmic, quadratic, and exponential models, and the p values for the trends compared. There was a significant trend across groups when the quadratic and exponential models were used (p=0.019 and p=0.015 respectively for MS, p=0.004 and p=0.033 respectively for unaffected siblings) (table 5.10 and figure 5.13).

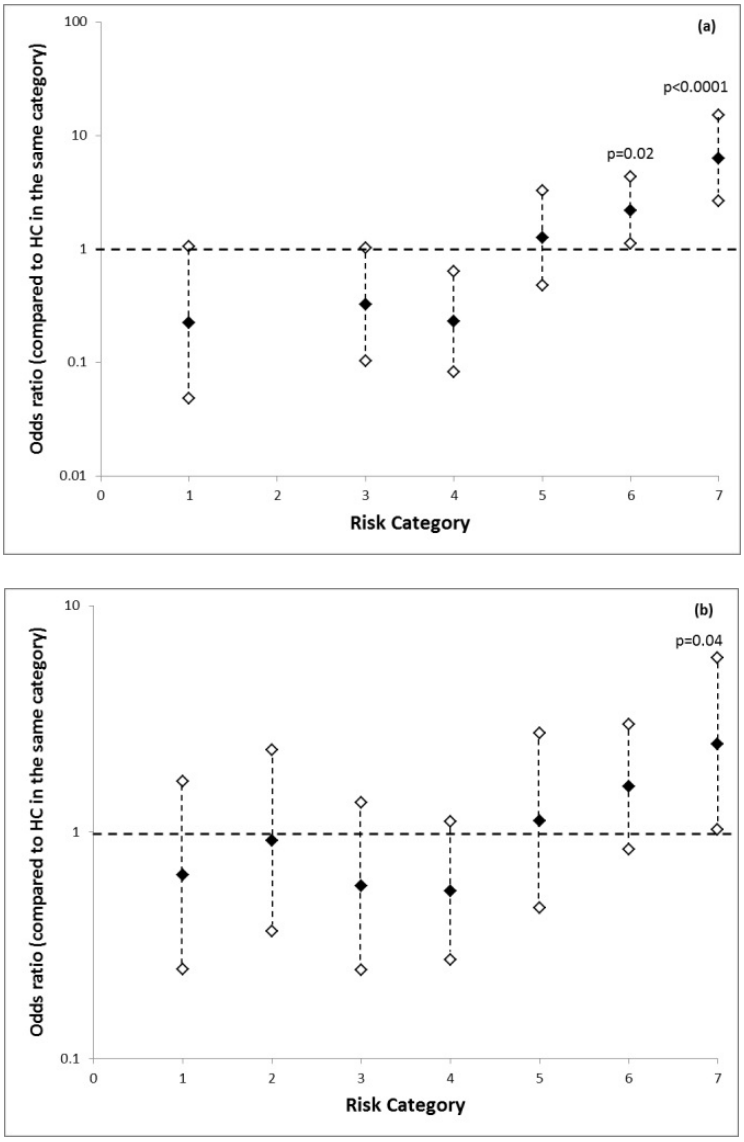


Figure 5.12: (a) OR of people with MS being in a given category of MS risk score compared to healthy controls. The solid diamond represents the calculated OR, and the line between the two unfilled diamonds the 95% confidence interval. **(b)** OR of the unaffected siblings being in a given category of MS risk score compared to healthy controls. The solid diamond represents the calculated OR, and the line between the two unfilled diamonds the 95% confidence interval.

Table 5.10: p values for the models used to assess the trend across OR for being in a given category of risk score compared to healthy controls

Model	p value (MS)	p value (Siblings)
Linear	0.064	0.026
Logarithmic	0.192	0.104
Quadratic	0.019	0.004
Exponential	0.015	0.033

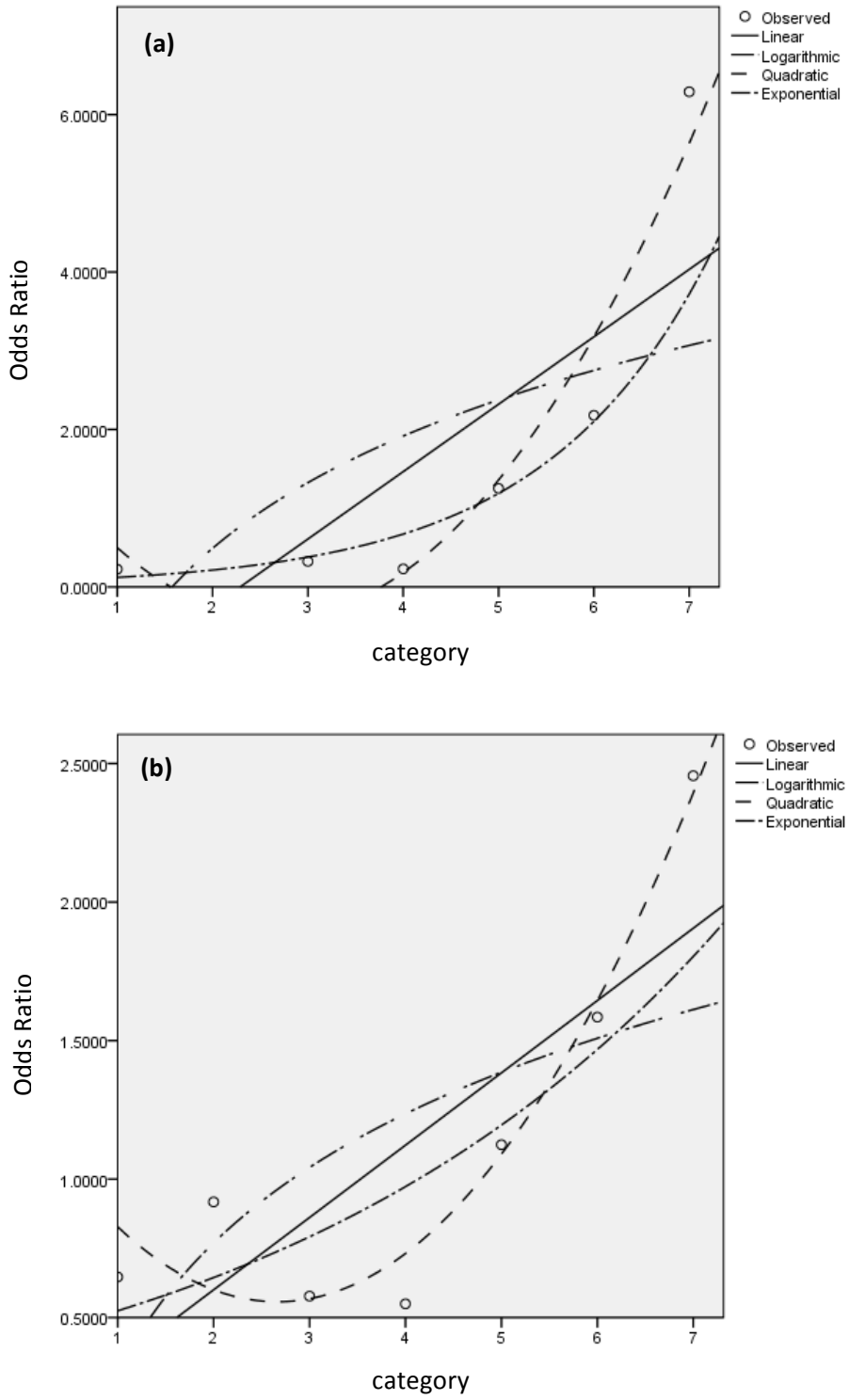


Figure 5.13: (a) Linear, logarithmic, quadratic and exponential models of OR across categories for the OR of people with MS being in a given category of MS risk score compared to healthy controls. **(b)** Linear, logarithmic, quadratic and exponential models for the OR of the unaffected siblings of people with MS being in a given category of MS risk score compared to healthy controls.

A second analysis was performed, again using the seven MS risk score categories described above. The probability of having MS (or of being an unaffected sibling of a person with MS) compared to the probability of being a healthy control subject for subjects in each category of the MS risk score was calculated and expressed as an odds ratio (table 5.11 and figure 5.14).

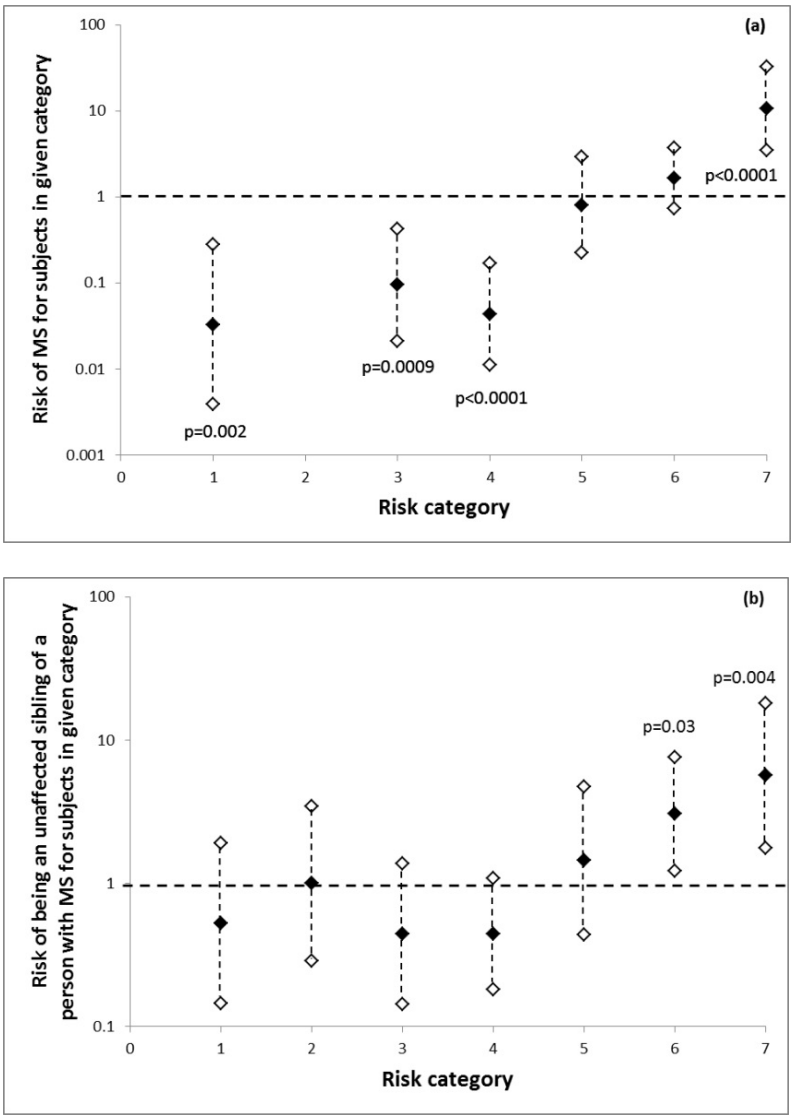


Figure 5.14: (a) OR of having MS for an individual in a given category of MS risk score. The solid diamond represents the calculated OR, and the line between the two unfilled diamonds the 95% confidence interval. **(b)** OR of being an unaffected sibling for a given category of MS risk score. The solid diamond represents the calculated OR, and the line between the two unfilled diamonds the 95% confidence interval.

Table 5.11: Odds ratio of either having MS, or being an unaffected sibling, compared to healthy control for each category of MS risk score.

	MS (OR; 95% CI)	Sibling (OR; 95% CI)
1	0.0331 (0.0039-0.2784)[a]	0.5289 (0.1459-1.9175)
2	N/A (0 patients)	1.000 (0.2895-3.4542)
3	0.0952 (0.0212-0.4281)[b]	0.444 (0.1433-1.3780)
4	0.0434 (0.0111-0.1695)[c]	0.444 (0.1817-1.0873)
5	0.8100 (0.2267-2.8946)	1.4400 (0.4395-4.7184)
6	1.6531 (0.7380-3.7029)	3.0476 (1.2276-7.5659)[d]
7	10.5625 (3.4441-32.3936)[c]	5.6406 (1.7538-18.1417)[e]

a: p=0.002; b: p=0.0009; c: p<0.0001; d: p=0.03; e: p=0.004

Again, the OR trend across the categories was modelled using for linear, logarithmic, quadratic, and exponential models, and the p values for the trends compared. There was a significant trend across the categories with an exponential model (p=0.012 and p=0.026 for MS and unaffected siblings respectively); and a significant trend for a quadratic model in the unaffected siblings (p=0.002) (table 5.12 and figure 5.15).

For those persons in the lowest category of MS risk score (i.e. category 1), there was a significantly reduced chance of having MS (OR 0.0331, 95%CI 0.0039-0.2784; p=0.002). This reduction in MS risk could be seen for the three lowest MS risk score categories. However, for these lower MS risk score categories there was no significant difference in the odds of being a sibling of a person with MS. For those individuals with risk scores in the highest MS risk score category (i.e. category 7), there was a significantly increased odds ratio of having MS compared to being a healthy control (OR 10.56, 95%CI 3.4441-32.3936; p<0.0001) and also a significantly increased odds ratio of being a sibling of a person with MS (OR 5.64, 95%CI 1.7538-18.1417; p=0.004). This highlights the potential utility of this risk score, as those with a low risk score are significantly less likely than chance to have MS; conversely those with a risk score in the highest category have a greatly increased odds ratio of having MS.

Table 5.12: p values for the models used to assess the trend across OR for having MS or being an unaffected sibling for each category

Model	p value (MS)	p value (Siblings)
Linear	0.124	0.025
Logarithmic	0.279	0.103
Quadratic	0.068	0.002
Exponential	0.012	0.026

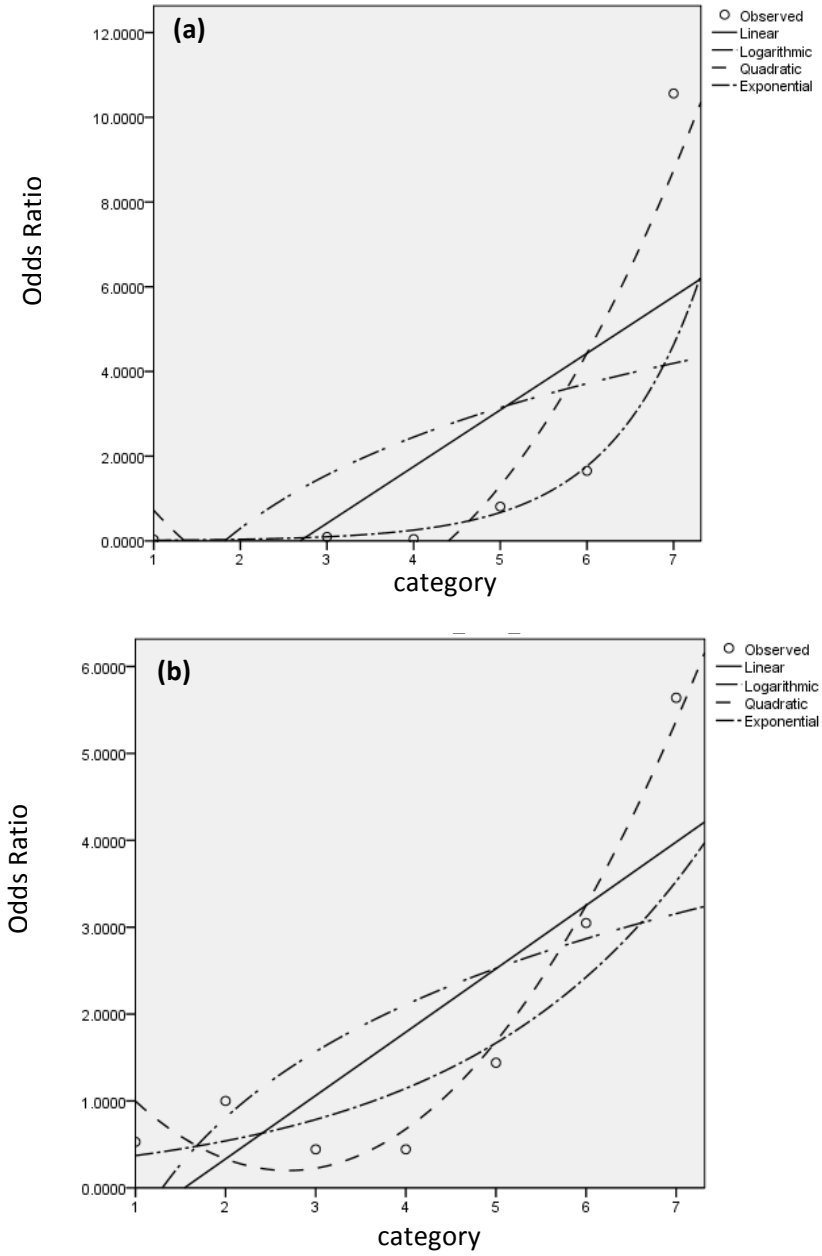


Figure 5.15: (a) Linear, logarithmic, quadratic and exponential models of OR across categories for the OR of people having MS for each category of MS risk score. **(b)** Linear, logarithmic, quadratic and exponential models for the OR of being an unaffected sibling of a person with MS for each category of MS risk score.

The odds ratio of being in the highest category of the risk score (i.e. category 7) compared to the lowest category of the risk score (i.e. category 1) was then calculated for each of the three categories (MS, unaffected siblings and healthy controls). People with MS had a significantly increased risk of being in the highest category (OR 169.00; 95% CI 22.11-1291.87; $p<0.00001$), where the odds of being in the lowest category of the risk score was taken as the reference category. There was a similar, but somewhat smaller, increase for unaffected siblings of people with MS (OR 5.64; 95% CI 1.75-18.14; $p=0.004$). Healthy controls were not more likely to be in one extreme than the other (OR of being in category 7 vs. category 1 0.62; 95% CI 0.21-1.89, $p=0.40$).

5.2.3. Including genetic contribution from all HLA and non-HLA SNPs identified by genome wide association studies

When the contribution from all MS risk alleles described by the 2011 GWAS (103) were included in the risk score calculation, the MS risk scores were significantly higher in all three groups than the scores with only HLA-DRB1*1501 included (table 5.8).

The risk scores for the three groups (people with MS, unaffected siblings and healthy controls) were normally distributed, and so parametric statistical methods were used to compare the groups. There was a significant difference overall between the MS risk scores of the three groups ($p<0.0005$; one-way ANOVA) (table 5.8 and figure 5.16). Post-hoc testing showed that there were significant differences between all pairwise combinations (table 5.8 and figure 5.16). The difference between the mean MS risk score of people with MS and their unaffected siblings was highly significant, as was the difference between people with MS and healthy controls ($p<0.0005$ for both comparisons). Similarly, the difference between the unaffected siblings of people with MS and healthy controls was highly significant ($p<0.0005$) (table 5.8 and figure 5.16).

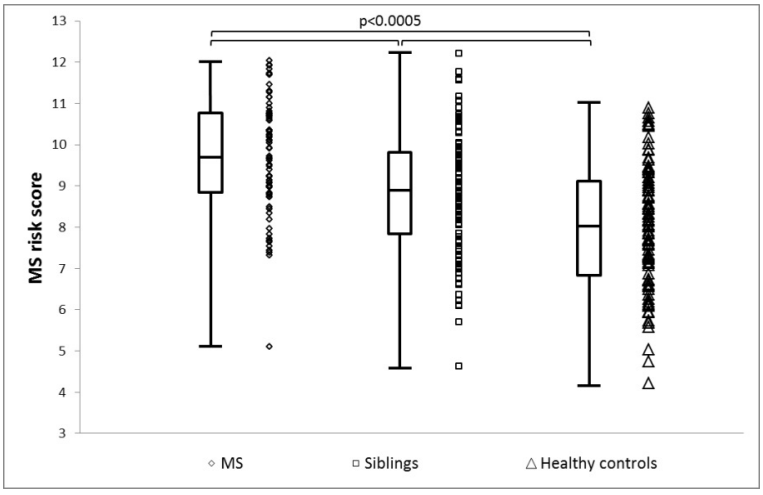


Figure 5.16: Combined scatter and box-and-whisker plot demonstrating the MS risk score distribution between people with MS, their unaffected siblings and healthy controls, where full genetic information is used to derive the MS risk score. The box indicates the interquartile range, bisected by the median, and the whiskers the range.

A ROC curve comparing people with MS with healthy controls generated an area under the curve of 0.801 (95%CI 0.735 – 0.866) (figure 5.17 and table 5.9).

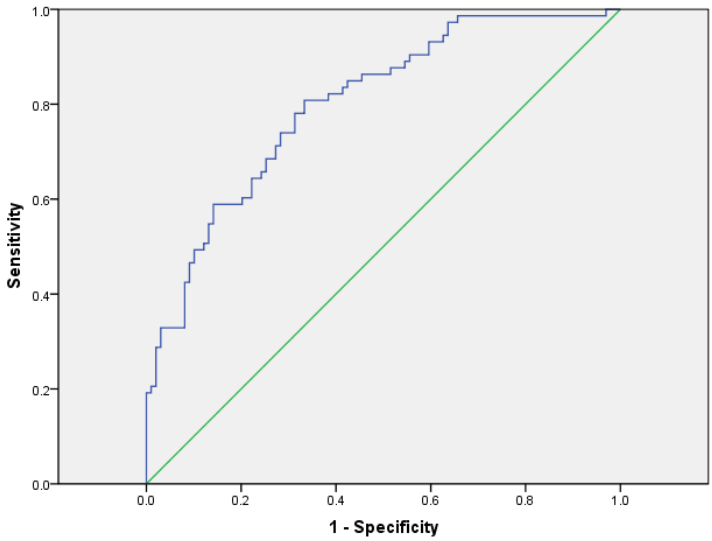


Figure 5.17: A receiver operating characteristic (ROC) curve generated by the MS risk score including full genetic information when the risk score of people with MS is compared to that of healthy controls.

As with the risk score generated using HLA-DRB1*1501 only, the MS risk score generated using the full genetic information was partitioned into seven categories using the distribution and values of the healthy control population. As previously, the seven categories were defined as +/- 0·25, 0·75, and 1·25 SD from the mean; the extreme categories were less than 1·25 or greater than 1·25 SD from the mean. The proportions of people with MS or siblings in a given category were compared to the proportion of healthy controls in that category (table 5.13 and figure 5.18). The trend for the OR across groups was modelled. The OR for both people with MS and unaffected siblings had a highly significant relationship when an exponential model was used ($p=0.001$ and $p<0.0005$ for people with MS and unaffected siblings respectively) (table 5.14).

Table 5.13: Odds ratio for being assigned to the categories defined using the healthy control MS risk score results, with the MS risk score derived using all genetic information. The proportion of people with MS and their unaffected siblings in each MS risk score category have been compared to the proportion of healthy control subjects in that category.

Category	MS (n)	MS (OR; 95% CI)	Sibling (n)	Siblings (OR; 95% CI)	Healthy control (n)
1	1	0.1007 (0.0128-0.7930)[a]	4	0.2816 (0.0876-0.9045)[a]	12
2	0	N/A (0 patients)	8	0.4906 (0.1964-1.2259)	14
3	4	0.3520 (0.1108-1.1179)	10	0.6259 (0.2643-1.4824)	14
4	7	0.4773 (0.1880-1.2116)	17	0.8500 (0.4106-1.7597)	18
5	13	1.1240 (0.5032-2.5106)	20	1.1925 (0.5788-2.4571)	16
6	12	1.3014 (0.5560-3.0461)	25	2.0169 (0.9668-4.2075)	13
7	36	7.0541 (3.3050-15.0558)[b]	23	1.9851 (0.9287-4.2431)	12

a: p=0.03; b: p<0.0001

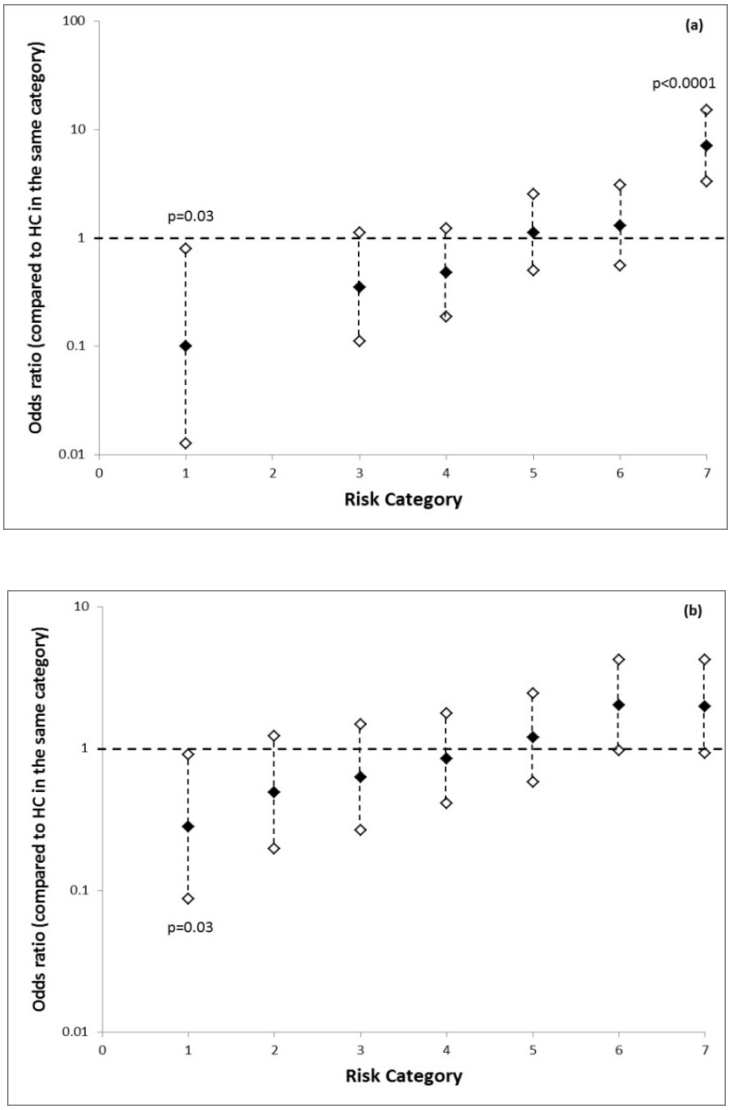


Figure 5.18: (a) OR of people with MS being in a given category of MS risk score compared to healthy controls, when full genetic information is used to calculate the MS risk score. The solid diamond represents the calculated OR, and the line between the two unfilled diamonds the 95% confidence interval. **(b)** OR of unaffected siblings being in a given category of MS risk score compared to healthy controls, where all genetic information is used to calculate the risk score. The solid diamond represents the calculated OR, and the line between the two unfilled diamonds the 95% confidence interval.

Table 5.14: p values for the models used to assess the trend across OR for being in a given category of risk score compared to healthy controls

Model	p value (MS)	p value (Siblings)
Linear	0.102	0.001
Logarithmic	0.237	0.009
Quadratic	0.075	0.003
Exponential	0.001	<0.0005

A further analysis was performed, again using the population partitioned into the seven categories described above. The probability of having MS (or of being an unaffected sibling of people with MS) compared to the probability of being a healthy control subject for the subjects in each MS risk score category was calculated and expressed as an odds ratio (table 5.15 and figure 5.19). There was a highly significant trend across the groups for an exponential model ($p<0.0005$ for exponential model for both people with MS and unaffected siblings) (table 5.16).

Table 5.15: Odds ratio of either having MS, or being an unaffected sibling, compared to healthy control for each category of MS risk score.

	MS (OR; 95% CI)	Siblings (OR; 95% CI)
1	0.0069 (0.0004-0.1243)[a]	0.1111 (0.0224-0.5505)[e]
2	N/A (0 patients)	0.3265 (0.0956-1.1154)
3	0.0816 (0.0170-0.3930)[b]	0.5102 (0.1619-1.6075)
4	0.1512 (0.0440-0.5198)[c]	0.8920 (0.3493-2.2776)
5	0.6602 (0.2345-1.8584)	1.5625 (0.6167-3.9590)
6	0.8521 (0.2809-2.5845)	3.6982 (1.4334-9.5414)[e]
7	9.0000 (3.5726-22.6727)[d]	3.6736 (1.3691-9.8575)[f]

a: p=0.0007; b: p=0.002; c: p=0.003; d: p<0.00001; e: p=0.007; f: p=0.010

Table 5.16: p values for the models used to assess the trend across OR for having MS or being an unaffected sibling for each category

Model	p value (MS)	p value (Siblings)
Linear	0.145	0.003
Logarithmic	0.303	0.024
Quadratic	0.099	0.005
Exponential	<0.0005	<0.0005

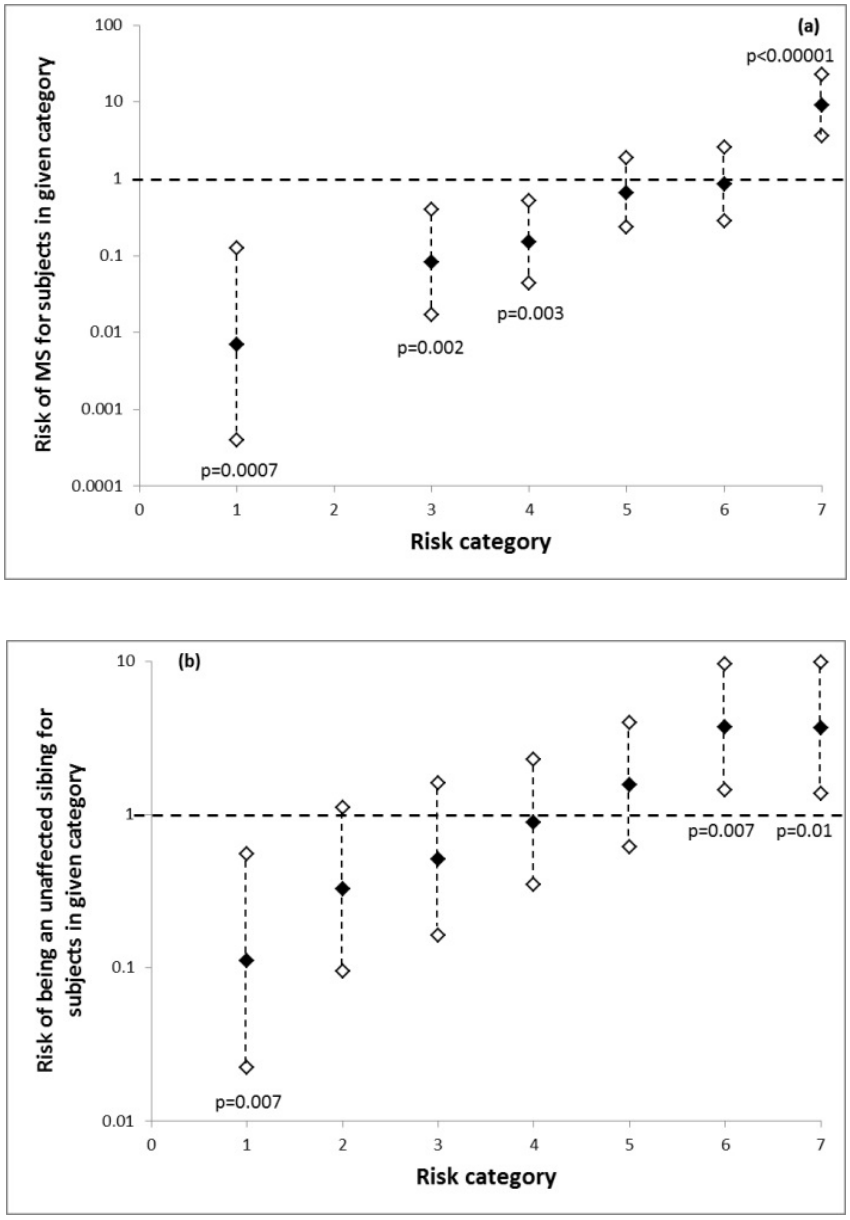


Figure 5.19: (a) OR of having MS for an individual in a given category of MS risk score, when full genetic information is used to derive the risk score. The solid diamond represents the calculated OR, and the line between the two unfilled diamonds the 95% confidence interval. **(b)** OR of being an unaffected sibling for a given category of MS risk score, when full genetic information is used to derive the risk score. The solid diamond represents the calculated OR, and the line between the two unfilled diamonds the 95% confidence interval.

Finally, the odds ratio of being in the highest category of the risk score compared to the lowest category of the risk score was calculated. People with MS had a significantly increased risk of being in the highest category of the risk score (OR 1296.00; 95% CI 78.02-21,527.34; p<0.00001), where

the odds of being in the lowest category of the risk score was taken as the reference category. There was a similarly highly significant, but somewhat smaller, increase for unaffected siblings of people with MS (OR 33.06; 95% CI 7.37-148.41; $p<0.00001$). Healthy controls were not more likely to be in one extreme than the other (OR of being in highest category vs. lowest category 1.00; 95% CI 0.32-3.10, $p=1.00$).

5.2.4. Effect of excluding serum 25-hydroxyvitamin D from calculations using HLA-DRB1*1501

Given the potential for post-diagnosis behavioural modification in terms of vitamin D supplementation in the MS group (discussed in section 5.1.2.6), a comparison of the risk scores excluding the contribution from serum 25-OHvD levels were also performed.

When the MS risk score calculated with the genetic contribution from HLA-DRB1*1501 only had the contribution from serum 25-OHvD levels excluded, the scores remained normally distributed. The overall significant difference between the risk scores remained ($p<0.0005$, one-way ANOVA) (figure 5.20 and table 5.8). However, whilst on post-hoc testing the significant difference between people with MS and their unaffected siblings ($p<0.0005$) and healthy controls ($p<0.0005$) remained, the difference between the mean risk score of unaffected siblings of people with MS and healthy controls was now of borderline significance ($p=0.053$) (figure 5.20 and table 5.8).

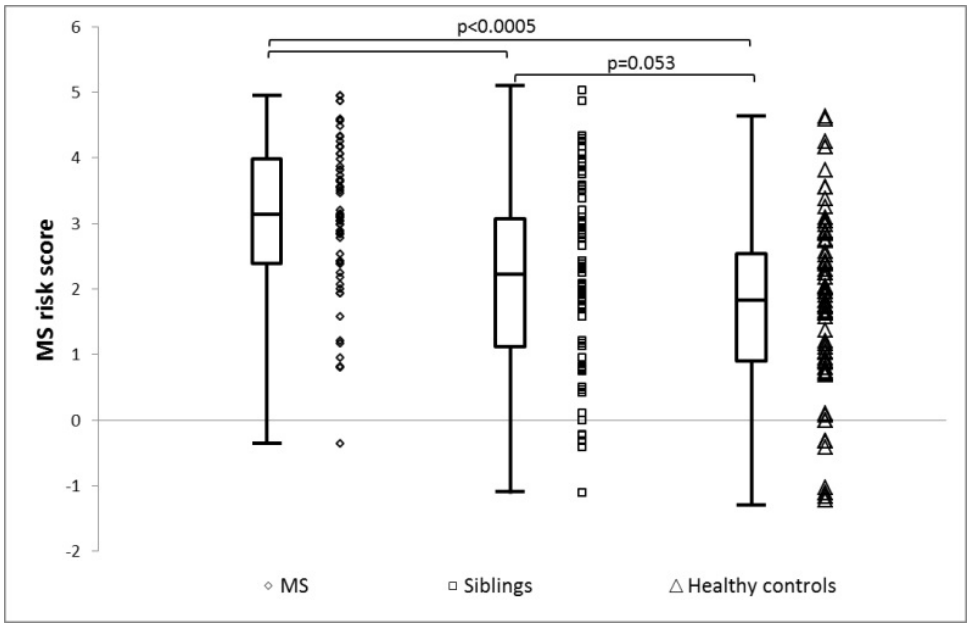


Figure 5.20: Combined scatter and box-and-whisker plot demonstrating the MS risk score distribution between people with MS, their unaffected siblings and healthy controls, where HLA-DRB1*1501 is used and serum 25-OHvD levels are excluded from the MS risk score derivation. The box indicates the interquartile range, bisected by the median, and the whiskers the range.

A ROC curve comparing people with MS with healthy controls generated an area under the curve of 0.800 (95% CI 0.733 – 0.868) (figure 5.21 and table 5.9).

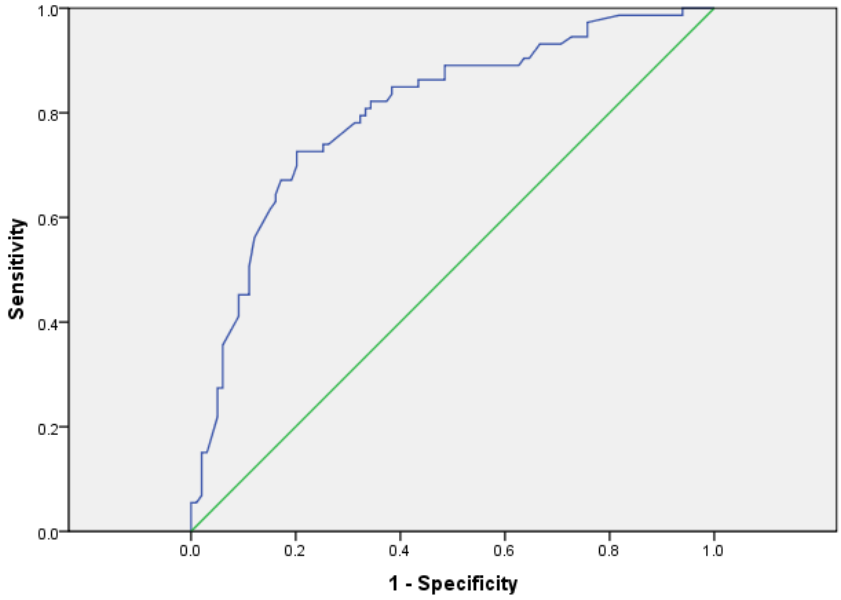


Figure 5.21: A ROC curve generated by the MS risk score including genetic information from HLA-DRB1*1501 only, and excluding serum 25-OHvD levels; the MS risk score of people with MS is compared to that of healthy controls.

Partitioning the risk score into seven categories gave similar results to those described previously. There was a significantly reduced odds of people with MS being in categories 1 and 4, with a borderline significant reduction in the risk of being in category 3. There was a significantly increased odds of people with MS being in categories 6 and 7, and of their unaffected siblings being in category 7 (table 5.17 and figure 5.22).

Table 5.17: Odds ratio for being assigned to the categories defined using the healthy control MS risk score results, with the MS risk score derived using HLA-DRB1*1501 and excluding serum 25-OHvD levels. The proportion of people with MS and their unaffected siblings in each MS risk score category have been compared to the proportion of healthy control subjects in that category.

Category	MS (n)	MS (OR; 95% CI)	Sibling (n)	Siblings (OR; 95% CI)	Healthy control (n)
1	1	0.0778 (0.0100-0.6033)[a]	10	0.5773 (0.2463-1.3532)	15
2	0	N/A (0 patients)	4	1.2427 (0.2711-5.6965)	3
3	6	0.4030 (0.1514-1.0727)[b]	16	0.7912 (0.3786-1.6533)	18
4	4	0.1716 (0.0568-0.5182)[c]	21	0.7228 (0.3743-1.3959)	25
5	9	0.8538 (0.3478-2.0958)	16	1.0675 (0.4914-2.3190)	14
6	20	2.2911 (1.0669-4.9201)[d]	18	1.2279 (0.5748-2.6232)	14
7	33	7.3425 (3.2997-16.3385)[e]	22	2.3035 (1.0305-5.1494)[f]	10

a: p=0.01; b: p=0.07; c: p=0.002; d: p=0.03; e: p<0.00001; f: p=0.04

The probability of having MS (or of being an unaffected sibling of people with MS) compared to the probability of being a healthy control subject for the subjects in each MS risk score category was then calculated and expressed as an odds ratio (table 5.17 and figure 5.22). There was a significant exponential trend across the OR for the groups (p=0.006 and p=0.052 for people with MS and unaffected siblings respectively) (table 5.18). Those participants in the lowest MS risk score groups had significantly lower odds of having MS, and those in the highest MS risk score group had significantly increased odds of having MS (OR 10.89) (table 5.19 and figure 5.23). Again, there was a significant exponential trend across the OR of people with MS (p=0.005) and a borderline significant exponential trend for their unaffected siblings (p=0.055) (table 5.20).

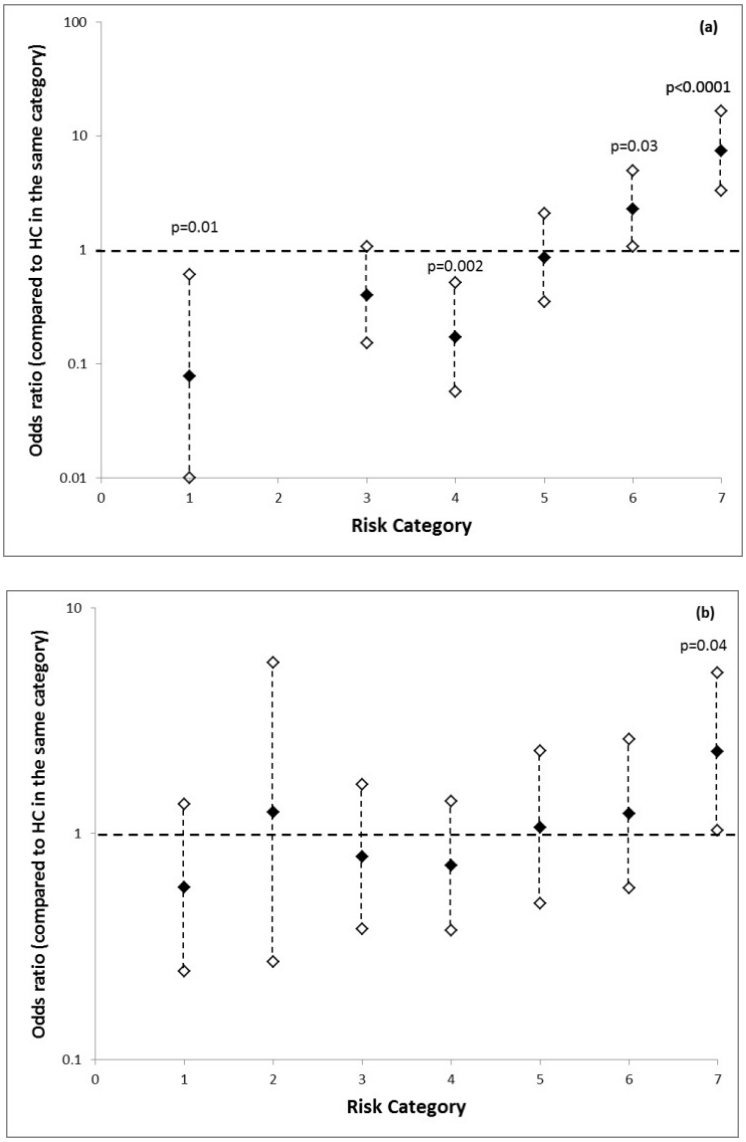


Figure 5.22: (a) OR of people with MS being in a given category of MS risk score compared to healthy controls, when HLA-DRB1*1501 is used, and serum 25-OHvD levels are excluded from the MS risk score calculation. The solid diamond represents the calculated OR, and the line between the two unfilled diamonds the 95% confidence interval. **(b)** OR of the unaffected siblings of people with MS being in a given category of MS risk score compared to healthy controls when HLA-DRB1*1501 is used, and serum 25-OHvD levels are excluded from the MS risk score calculation. The solid diamond represents the calculated OR, and the line between the two unfilled diamonds the 95% confidence interval.

Table 5.18: p values for the models used to assess the trend across OR for being in a given category of risk score compared to healthy controls

Model	p value (MS)	p value (Siblings)
Linear	0.078	0.064
Logarithmic	0.212	0.136
Quadratic	0.030	0.068
Exponential	0.006	0.052

Table 5.19: Odds ratio of either having MS, or being an unaffected sibling, compared to healthy control for each category of MS risk score.

	MS (OR; 95% CI)	Siblings (OR; 95% CI)
1	0.0044 (0.0003-0.0778)[a]	0.4444 (0.1433-1.3780)
2	N/A (0 patients)	1.7778 (0.2140-14.7666)
3	0.1111 (0.0301-0.4104)[b]	0.7901 (0.3048-2.0479)
4	0.0256 (0.0058-0.1139)[c]	0.7056 (0.3106-1.6028)
5	0.4133 (0.1264-1.3506)	1.3061 (0.4736-3.6018)
6	2.0408 (0.7768-5.3614)	1.6531 (0.6156-4.4387)
7	10.8900 (4.0040-29.6185)[d]	4.8400 (1.6817-13.9299)[e]

a: p=0.0002; b: p=0.001; c: p<0.00001; d: p<0.00001; e: p=0.003

Table 5.20: p values for the models used to assess the trend across OR for having MS or being an unaffected sibling for each category

Model	p value (MS)	p value (Siblings)
Linear	0.121	0.083
Logarithmic	0.278	0.176
Quadratic	0.059	0.063
Exponential	0.005	0.055

People with MS had a significantly increased risk of being in the highest category of the risk score (OR 59.4; 95% CI 7.83-450.76; p<0.0001), where the odds of being in the lowest category of the risk score was taken as the reference category. There was a similar, but somewhat smaller, increase for unaffected siblings of people with MS (OR 2.51; 95% CI 1.13-5.60; p=0.02). Healthy controls were not more likely to be at one extreme than the other (OR of being in highest category vs. lowest category 0.6292; 95% CI 0.27-1.48, p=0.29).

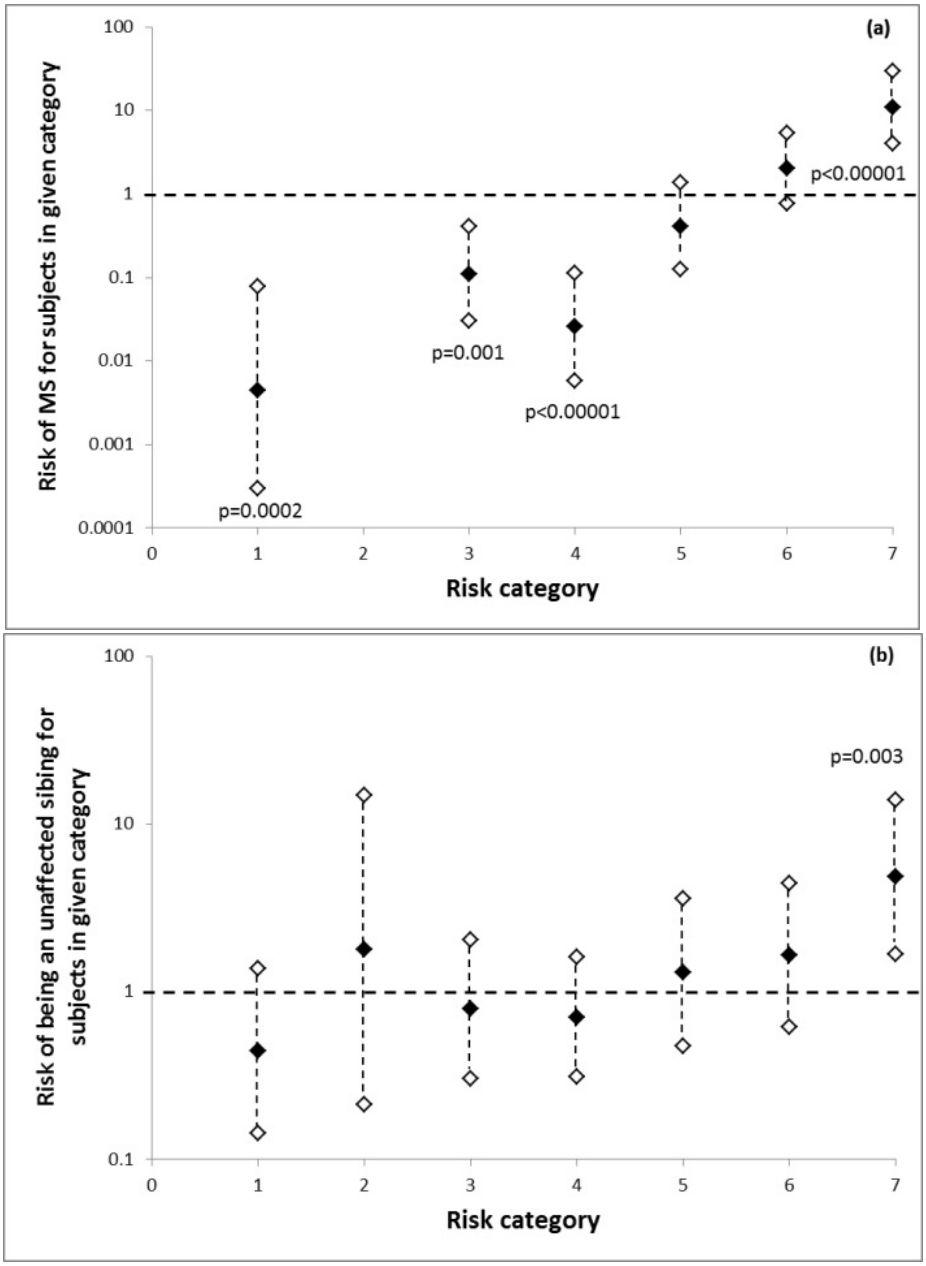


Figure 5.23: (a) OR of having MS for an individual in a given category of MS risk score, when HLA-DRB1*1501 is used, and serum 25-OHvD levels are excluded from the MS risk score calculation. The solid diamond represents the calculated OR, and the line between the two unfilled diamonds the 95% confidence interval. **(b)** OR of being an unaffected sibling for a given category of MS risk score, when HLA-DRB1*1501 is used, and serum 25-OHvD levels are excluded from the MS risk score calculation. The solid diamond represents the calculated OR, and the line between the two unfilled diamonds the 95% confidence interval.

5.2.5. Effect of excluding serum 25-hydroxyvitamin D from calculations including full genetic data.

When the MS risk score with all genetic information had the contribution from serum 25-OHvD levels excluded, the MS risk scores of the three groups remained normally distributed. There was a significant difference overall between the MS risk scores of the three groups ($p<0.0005$, one-way ANOVA) (table 5.8 and figure 5.24). Post-hoc testing showed that the differences were significant between all pairwise combinations (table 5.8 and figure 5.24). The difference between the mean MS risk score of people with MS and their unaffected siblings was highly significant, as was the difference between people with MS and healthy controls ($p<0.0005$ for both comparisons). Similarly, the difference between the unaffected siblings of people with MS and healthy controls was highly significant ($p<0.0005$) (table 5.8 and figure 5.24).

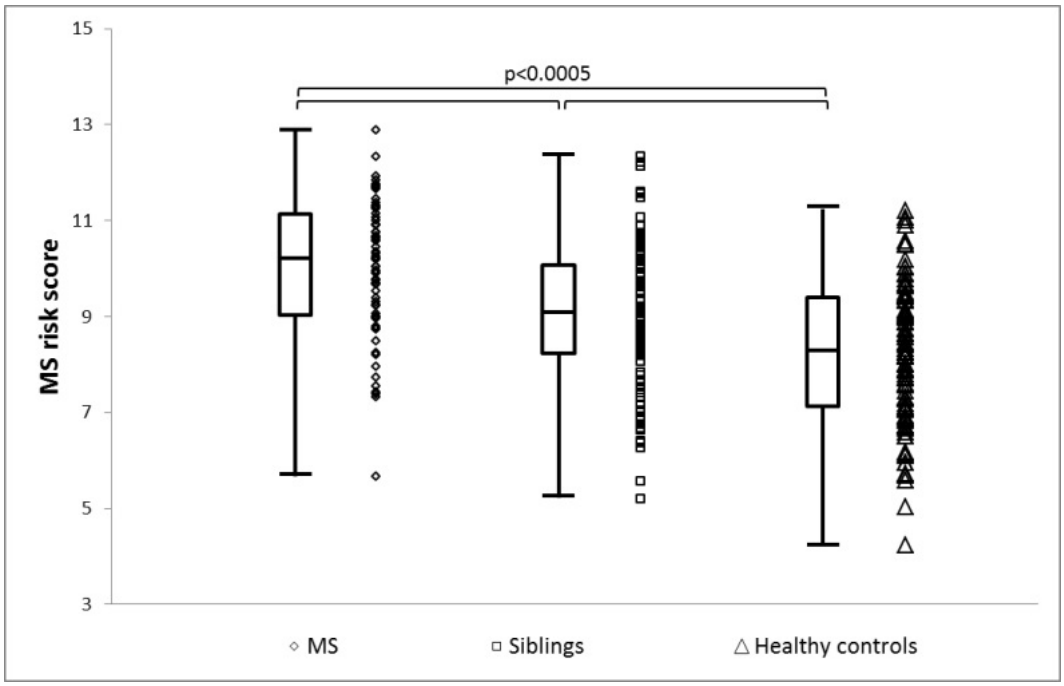


Figure 5.24: Combined scatter and box-and-whisker plot demonstrating the MS risk score distribution between people with MS, their unaffected siblings and healthy controls, where full genetic information is used and serum 25-OHvD levels are excluded from the MS risk score derivation. The box indicates the interquartile range, bisected by the median, and the whiskers the range.

A ROC curve comparing people with MS with healthy controls generated an area under the curve of 0.818 (95% CI 0.754 – 0.881) (figure 5.25 and table 5.9).

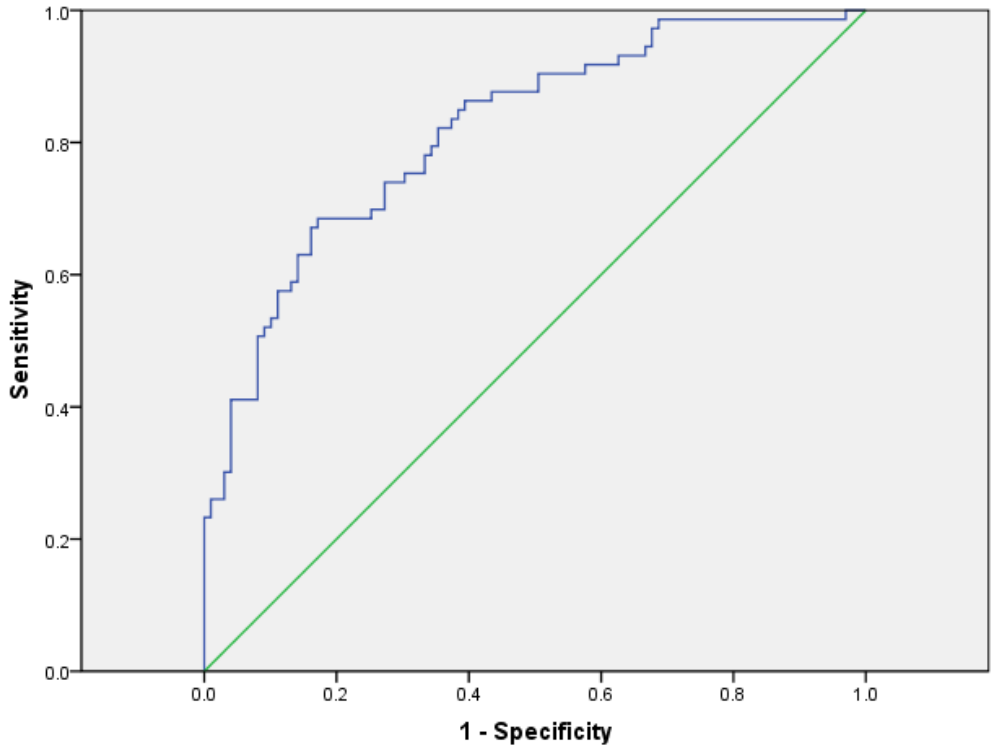


Figure 5.25: A ROC curve generated by the MS risk score including full genetic information, and excluding serum 25-OHvD levels; the MS risk score of people with MS is compared to that of healthy controls.

Again, partitioning the risk score into seven categories revealed significantly reduced odds of people with MS being in categories 1 and 4 with significantly increased odds of being in category 7. Unaffected siblings had significantly reduced odds of being in category 1 and significantly increased odds of being in category 7 (table 5.21 and figure 5.26). A significant exponential trend was seen in both the MS and unaffected siblings groups (p=0.015 and p=0.003 respectively) (table 5.22).

Those participants in the lowest MS risk score categories had significantly lower odds of having MS or being an unaffected sibling of someone with MS, and those in the highest MS risk score group had

significantly increased odds of having MS (OR 17.8) or being a sibling of a person with MS (OR 7.7) (table 5.23 and figure 5.27). Again, the exponential model showed the best relationship with the trend for the OR over the categories (p=0.009 for people with MS and p=0.003 for unaffected siblings) (table 5.24). People with MS had a significantly increased risk of being in the highest category of the risk score vs. the lowest category (OR 1444.00; 95% CI 87.11-23,937.80; p<0.00001). Unaffected siblings also had an increased risk (OR 69.44; 95% CI 12.77-377.71; p<0.00001). Healthy controls were not more likely to be at one extreme than the other (OR of being in highest category vs. lowest category 1.49; 95% CI 0.43-5.19, p=0.53).

Table 5.21: Odds ratio for being assigned to the categories defined using the healthy control MS risk score results, with the MS risk score derived using full genetic information and excluding serum 25-OHvD levels. The proportion of people with MS and their unaffected siblings in each MS risk score category have been compared to the proportion of healthy control subjects in that category.

Category	MS (n)	MS (OR; 95% CI)	Sibling (n)	Siblings (OR; 95% CI)	Healthy control (n)
1	1	0.1111 (0.0140-0.8811)[a]	3	0.2308 (0.0624-0.8534)[d]	11
2	0	N/A (0 patients)	9	0.6075 (0.2475-1.4911)	13
3	5	0.4118 (0.1425-1.1902)	10	0.5773 (0.2463-1.3532)	15
4	4	0.2609 (0.0843-0.8076)[b]	16	0.7912 (0.3786-1.6533)	18
5	12	1.1016 (0.4815-2.5204)	24	1.6193 (0.7938-3.3030)	15
6	13	0.9750 (0.4435-2.1432)	20	1.0345 (0.5111-2.0937)	18
7	38	10.8571 (4.7587-24.7707)[c]	25	3.0488 (1.3447-6.9122)[e]	9

a: p=0.04; b: p=0.02; c: p<0.00001; d: p=0.03; e: p=0.008

Table 5.22: p values for the models used to assess the trend across OR for being in a given category of risk score compared to healthy controls

Model	p value (MS)	p value (Siblings)
Linear	0.149	0.018
Logarithmic	0.304	0.055
Quadratic	0.112	0.040
Exponential	0.015	0.003

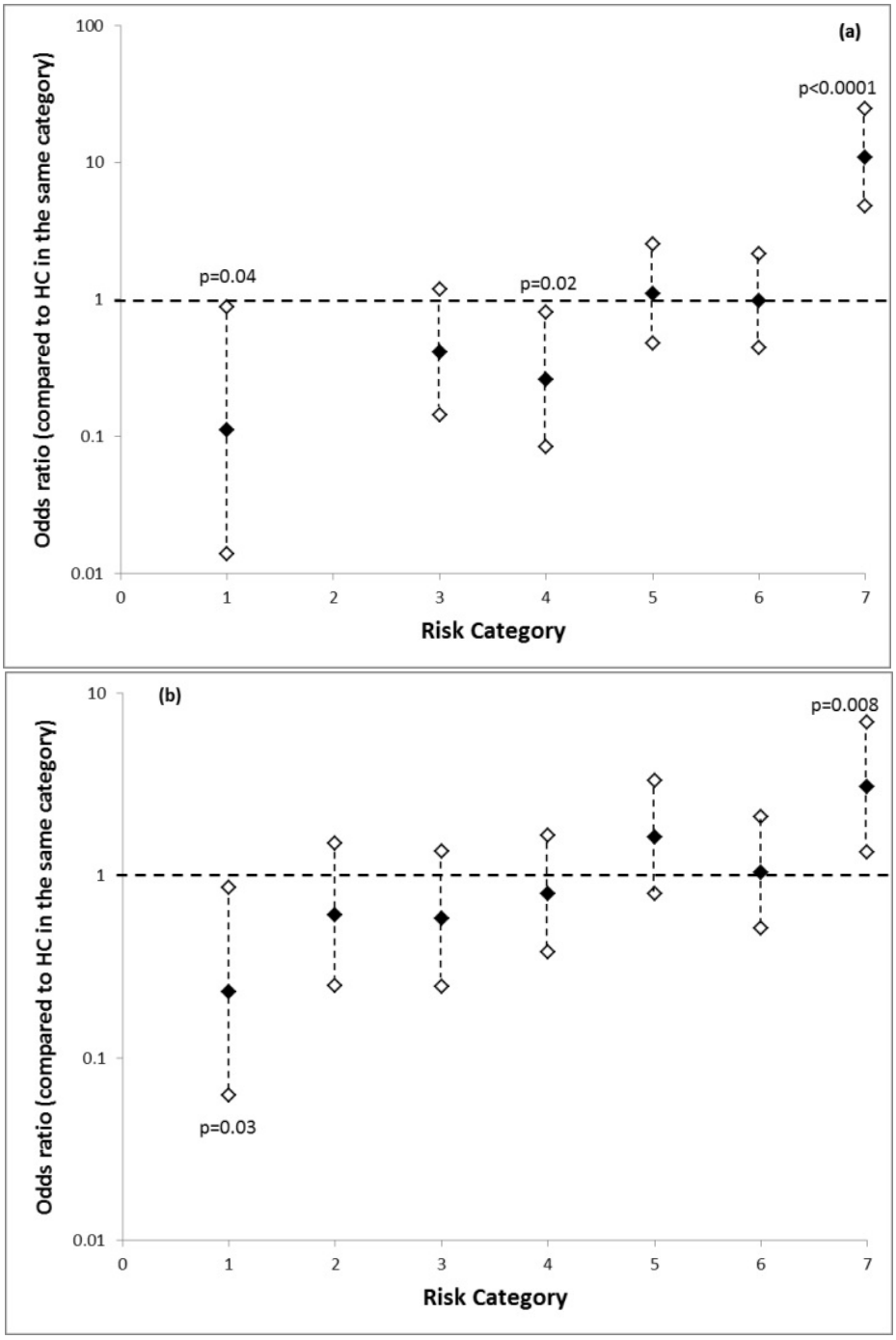


Figure 5.26: (a) OR of people with MS being in a given category of MS risk score compared to healthy controls, when full genetic information is used, and serum 25-OHvD levels are excluded from the MS risk score calculation. The solid diamond represents the calculated OR, and the line between the two unfilled diamonds the 95% confidence interval. **(b)** OR of the unaffected siblings of people with MS being in a given category of MS risk score compared to healthy controls when full genetic information is used, and serum 25-OHvD levels are excluded from the MS risk score calculation. The solid diamond represents the calculated OR, and the line between the two unfilled diamonds the 95% confidence interval.

Table 5.23: Odds ratio of either having MS, or being an unaffected sibling, compared to healthy control for each category of MS risk score.

	MS (OR; 95% CI)	Siblings (OR; 95% CI)
1	0.0083 (0.0005-0.1495)[a]	0.0744 (0.0122-0.4524)[e]
2	N/A (0 patients)	0.4793 (0.1441-1.5944)
3	0.1111 (0.0266-0.4649)[b]	0.4444 (0.1433-1.3780)
4	0.0494 (0.0107-0.2286)[c]	0.7901 (0.3048-2.0479)
5	0.6400 (0.2188-1.8724)	2.5600 (1.0281-6.3746)[f]
6	0.5216 (0.1902-1.4305)	1.2346 (0.5017-3.0382)
7	17.8272 (6.3801-49.8124)[d]	7.7160 (2.6269-22.6642)[g]

a: p=0.001; b: p=0.003; c: p=0.0001; d: p<0.00001; e: p=0.005; f: p=0.04; g: p=0.0002

Table 5.24: p values for the models used to assess the trend across OR for having MS or being an unaffected sibling for each category

Model	p value (MS)	p value (Siblings)
Linear	0.182	0.047
Logarithmic	0.350	0.122
Quadratic	0.131	0.050
Exponential	0.009	0.003

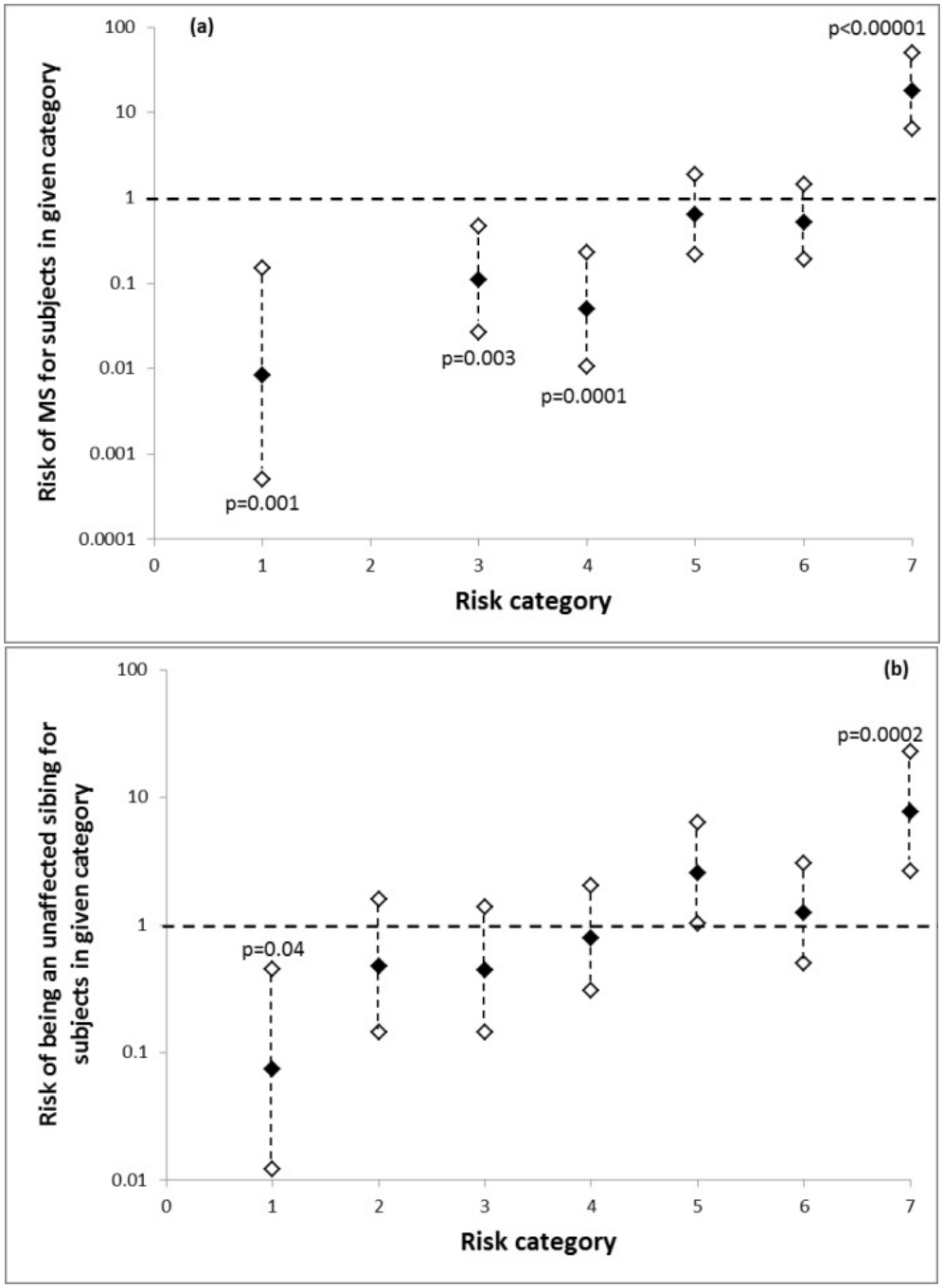


Figure 5.27: (a) OR of having MS for an individual in a given category of MS risk score, when full genetic information is used, and serum 25-OHvD levels are excluded from the MS risk score calculation. The solid diamond represents the calculated OR, and the line between the two unfilled diamonds the 95% confidence interval. **(b)** OR of being an unaffected sibling for a given category of MS risk score, when full genetic information is used, and serum 25-OHvD levels are excluded from the MS risk score calculation. The solid diamond represents the calculated OR, and the line between the two unfilled diamonds the 95% confidence interval.

5.2.6. Effect of excluding serum 25-hydroxyvitamin D levels on MS risk scores of unaffected siblings

Given the significant effect of post-diagnosis behavioural modification on serum 25-OHvD levels, there was concern that the improved area under the ROC curve seen when following the excluding of serum 25-OHvD levels would not translate into an improvement when the model was used as a prognostic tool in the unaffected siblings. There was no evidence that in this cohort the unaffected siblings had modified their behaviour in response to the diagnosis of MS in their affected siblings, as they reported vitamin D supplementation at a similar level to healthy controls.

In order to attempt to explore the potential effect of excluding serum 25-OHvD levels from the model, a number of post-hoc analyses were performed. The MS risk score calculated using genetic information from HLA-DRB1*1501 only was used in this analysis, in order to ensure that the contribution from serum 25-OHvD levels towards the final score was the largest possible proportion of the overall MS risk score. The unaffected siblings of people with MS were put into rank order using the MS risk score. They were then divided by the median MS risk score value for the group into a putative high and low risk cohort. The serum 25-OHvD levels between these two groups were then compared. The mean serum 25-OHvD level of the siblings with in the lower MS risk score cohort was 56.11nmol/l, compared to 56.22nmol/l for those siblings in the higher MS risk score cohort (difference not significant) (figure 5.28a). In order to ensure that the lack of difference was not caused by subjects with MS risk scores around the median, the serum 25-OHvD levels of the 20 siblings with the highest MS risk scores were compared to the serum 25-OHvD levels of the 20 siblings with the lowest MS risk scores (figure 5.28b). Again, there was no difference between the 20 siblings with the highest MS risk score (mean serum 25-OHvD level 54.67nmol/l) and the 20 siblings with the lowest MS risk score (mean serum 25-OHvD level 57.62nmol/l). Finally, the serum 25-OHvD levels in each category of the MS risk score (i.e. categories 1-7) were compared. There was no

significant difference in the mean MS risk scores between the categories when these were compared with a one-way ANOVA (figure 5.29).

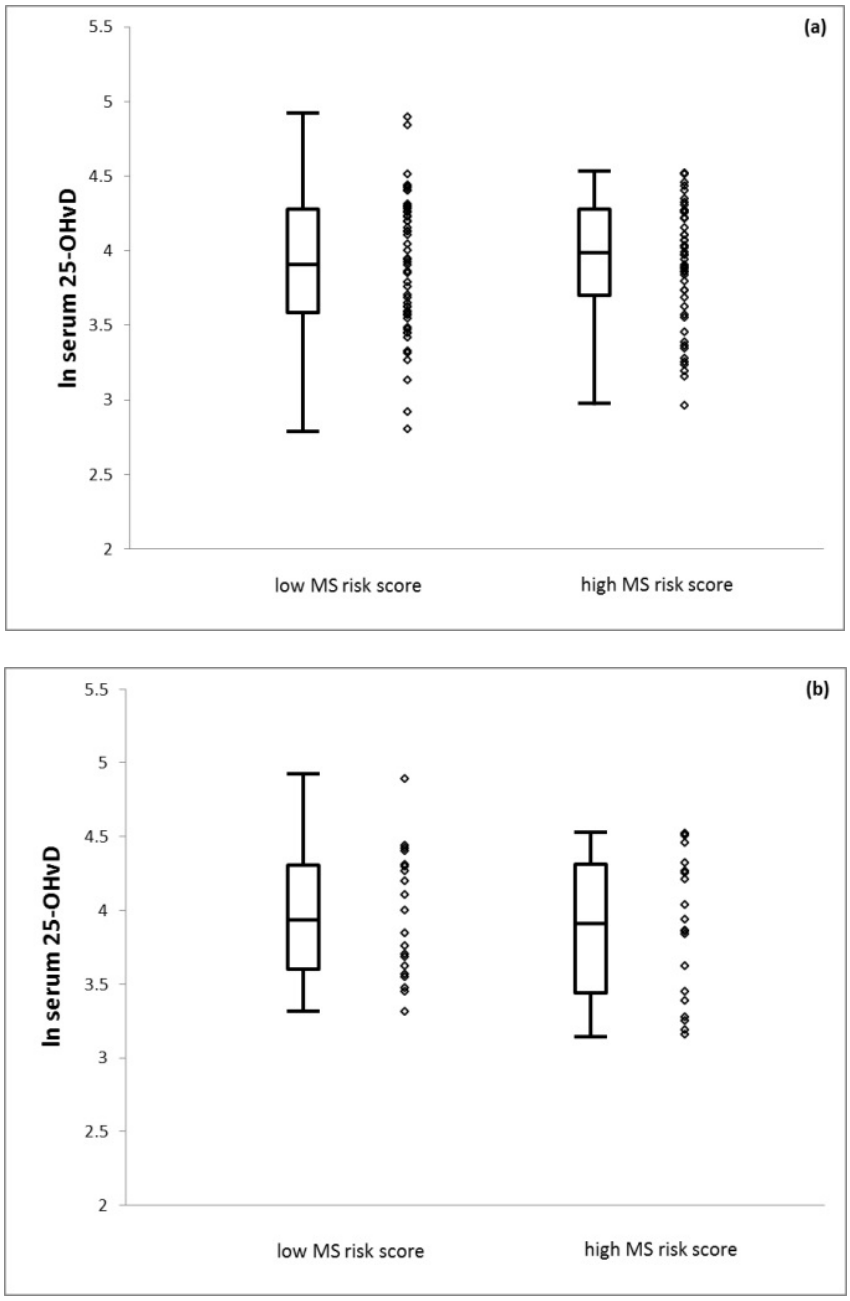


Figure 5.28: (a) Combined scatter and box-and-whisker plot demonstrating the lack of difference in serum 25-OHvD levels between those unaffected siblings with MS risk scores greater than the group median, and those with scores lower than the group median. The box indicates the interquartile range, bisected by the median, and the whiskers the range. **(b)** Combined scatter and box-and-whisker plot demonstrating the lack of difference in serum 25-OHvD levels between those unaffected siblings with highest MS risk scores and those with the lowest MS risk scores. The box indicates the interquartile range, bisected by the median, and the whiskers the range.

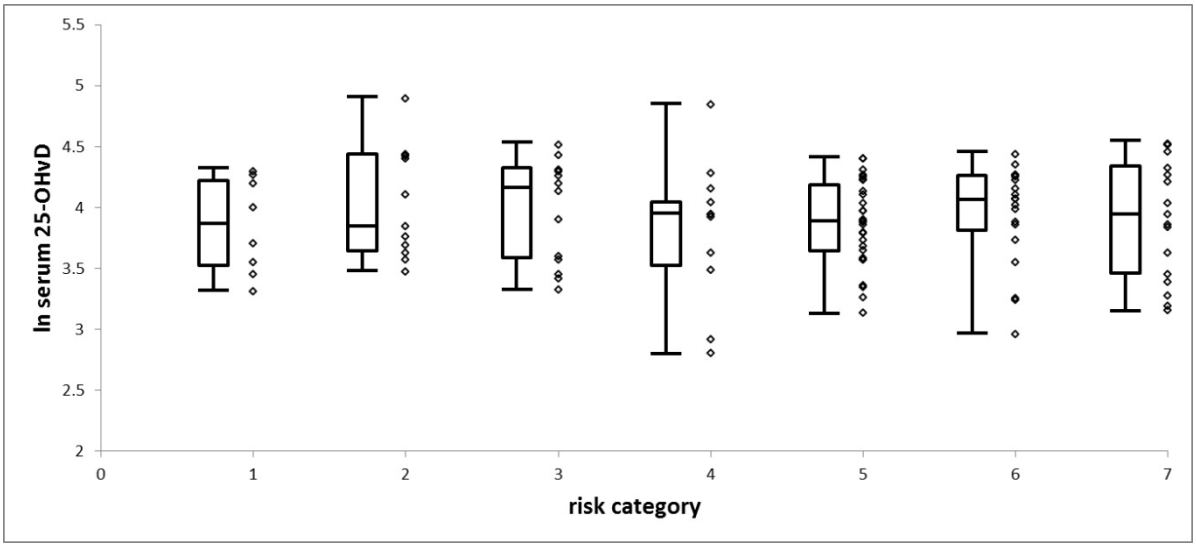


Figure 5.29: Combined scatter and box-and-whisker plot demonstrating the lack of difference in serum 25-OHvD levels over the 7 categories of MS risk score (defined using the range of healthy control risk scores). The box indicates the interquartile range, bisected by the median, and the whiskers the range.

A final analysis was performed whereby the individuals with the 20 highest and lowest MS risk scores were determined both with serum 25-OHvD levels included and excluded from the MS risk score calculation. The individuals identified using each method were then compared in order to assess the overlap in individuals identified using each method. 18/20 individuals for both the lowest and highest MS risk scores were the same regardless of whether serum 25-OHvD levels were included in the risk score calculation or not. The similarity between the two groups highlights the relatively small contribution of serum 25-OHvD to the overall MS risk score – the greatest modulation of MS risk has been shown to occur at extreme levels of serum 25-OHvD (147), and very few siblings in this study had serum 25-OHvD levels approaching this level, hence the apparent lack of effect when this is excluded from the risk score calculation.

5.2.7. Conclusions

It can therefore be seen that the putative MS risk score shows good discrimination between people with MS and healthy controls. It is not surprising that the inclusion of the full genetic information improves the MS risk score considerably; although the contribution to overall MS risk from each of the SNPs identified is relatively small, the overall contribution to risk is much greater.

Removal of serum 25-OHvD levels from the MS risk score calculation improved the AUC to a value approaching clinical utility – 0.818. This is far superior to previous attempts to develop such a risk score, which were able to achieve an AUC of 0.69 (275) and 0.72 (157). A value of 0.85 is generally taken as the level at which a test becomes potentially clinically useful (157). It is not clear whether inclusion of serum 25-OHvD levels in a population that had not undergone post-diagnosis behavioural modification would improve the AUC further; it could be supposed that this might be the case. An analysis excluding the patients who reported regularly supplementing with vitamin D was not performed, as by excluding these 15 patients the number of patients included in the analysis became so small as to widen the 95% confidence intervals to an unacceptable degree.

For those participants with the highest MS risk score, the odds of having MS vs. not having MS was in the region of 10, regardless of the risk score calculated. Similarly, for those with the lowest risk score, the risk of MS was significantly reduced. This highlights the potential utility of the risk score from a clinical point of view – whilst it is more difficult to interpret an intermediate score, a high score is associated with a far higher risk of MS. The most robust trend of the odds ratios with respect to the MS risk score categories appeared to be an exponential one, proving significant for all iterations of the putative MS risk score. This highlights the utility of this risk score – those with risk scores at the highest extreme have a highly significantly increased risk of MS. Interestingly, an similar exponential trend could be seen for the unaffected siblings of people with MS for all iterations of the MS risk score bar one – that where serum 25-OHvD levels were excluded from the calculation and

only the genetic information from HLA-DRB1*1501 was used. Despite the fact that all other robustness analysis demonstrated no significant effect of excluding serum 25-OHvD levels from the calculations, this loss of the trend seen in all other analyses hints at a potentially important effect of serum 25-OHvD in the generation of MS risk, prior to clinical presentation.

Clearly further work is required to validate the suggested MS risk score. Firstly, a larger cohort of people with MS and healthy controls is required in order to gather more precise estimates of the odds ratios associated with extreme risk scores, and to narrow the confidence interval of the area under the curve. Whether the paradigm enshrined within the MS risk score holds true for those at risk of MS, who have not yet developed clinical disease has yet to be investigated.

In the next part of this work, those unaffected siblings with high MS risk scores will be compared to those siblings with low risk score, in order to determine if any difference can be determined between the two groups, both in terms of biomarkers previously associated with MS and also MRI evidence of subclinical disease.

Chapter 6: Potential markers of the endophenotype

6.1. Autoimmune disease in siblings

6.1.1. Background

The study of the risk of additional autoimmune diseases both in people with MS, and their first-degree relatives has been pursued over many years, with studies employing a variety of designs and yielding conflicting results (282). As part of this sibling study, both people with MS and their siblings were questioned about co-morbidities, including autoimmune diseases. Whilst this represents a relatively small-scale contribution to the literature on autoimmune disease in MS, the opportunity to directly question siblings of people with MS regarding their comorbidities provided an interesting avenue of study.

The most recent large-scale study to attempt to address this question (283) used the Swedish National MS register together with the Swedish National Patient Register. Roshanisefat et al (283) found no consistent evidence for an increased risk of autoimmune disease in the parents of people with MS; additionally the authors found that the risk of a second autoimmune disease appeared to be increased only after the diagnosis of MS (283). This finding, which suggests that the increased risk seen in MS may be a result of the increased contact that people with MS have with healthcare professionals, implies that there may be either surveillance or reporting bias underlying previous reports of an increased risk of additional diagnoses.

However, this study (283) is not the only one using a national database in order to attempt to answer the question regarding MS and autoimmune disease. National databases from Denmark (284, 285), the UK (286), California (287) and Taiwan (288) have also been employed to address this question.

6.1.2. Defining rates of autoimmune disease in the UK population

When examining rates of autoimmune disease in people with MS and their relatives, it is important that the population rates used as a comparator are as accurate as possible. Figures taken from one of the most commonly used databases in the UK, the Hospital Episodes Statistics (HES) database carry a considerable risk of overestimating the population risk. The HES database uses data from hospital inpatients only, and so does not capture those members of the population who do not attend hospital. It is therefore likely that population rates of autoimmune disease are over-estimated in this database. The UK General Practice research database does not sample the entire country, and relies on the accuracy of coding within individual practices, meaning that some disease may be significantly over- or under-estimated within this database.

Another important consideration is that rates of autoimmune disease may differ considerably between males and females. Overall population rates merely provide an average rate, and when comparing populations where the gender distribution is not identical between the populations under study (as in this cohort), gender-specific disease rates must be used where there is a difference in male and female rates of the disease in question.

Overall and gender-specific disease rates were therefore established from the published literature for a variety of autoimmune and other diseases. The non-autoimmune diseases examined (type 2 diabetes and seizures) were included as it was felt important to have a comparator to the autoimmune diseases. Hypothyroidism was used as a surrogate marker for autoimmune thyroid disease as there is good evidence that the majority of hypothyroidism in iodine-replete areas is autoimmune in nature (289). The same cannot be said of hyperthyroidism, and so this was not used as a surrogate in the same way.

Details regarding the co-morbid diagnoses in both people with MS and their unaffected siblings was gathered using the structured questionnaire provided in appendix 4. Participants were directly asked

if they had any past medical history of thyroid disorders, diabetes, arthritis, lupus, psoriasis, gastrointestinal problems or if they had ever had a seizure. If they answered yes to any of these questions, further questions were asked regarding both the precise diagnosis (if known), the investigations that they had undergone, and any treatment that they had received. In the case of diabetes, participants were questioned regarding the use of insulin and oral therapies, together with the age of diagnosis. From this information, it was possible to infer a diagnosis for those participants who were unsure as to their precise diagnostic label. In addition, participants were all asked to provide a full list of medication. If any participants were taking medications such as thyroxine, they were questioned further regarding this. If participants reported regular B12 injections then they were questioned further regarding this. Regular B12 injections were assumed to infer a diagnosis of pernicious anaemia. Whilst this approach may have led to an overestimation of certain diseases in the MS and sibling population, the use of close questioning regarding both concomitant medication and prior investigations performed was felt to keep this to a minimum. GPs were not contacted regarding comorbid diagnoses and the GP records of participants were not accessed due to ethical concerns.

Details of the diseases studied, background population rates and sources of data are given in table 6.1.

Table 6.1: Background population and gender specific rates of selected autoimmune and non-autoimmune diseases.

Disease	UK population prevalence (cases per 10,000)	UK Female population prevalence (cases per 10,000)	UK Male population prevalence (cases per 10,000)	Literature sources
Hashimotos/hypothyroidism	80	170	20	(290-293)
Type 1 diabetes	34	34	34	(291, 292, 294, 295)
Type 2 diabetes	450	340	430	(296)
Rheumatoid arthritis	55	107	47	(291, 292, 297)
Pernicious anaemia	13	13	13	(291, 292, 297)
Systemic lupus erythematosus (SLE)	2.7	4.8	0.5	(291, 292, 298, 299)
Psoriasis	150	150	150	(300)
Coeliac disease	5	5.4	4.7	(297)
Inflammatory bowel disease	26	26	26	(301)
Seizures	75	75	75	(302)

6.1.3. Autoimmune disease rates in siblings of people with MS

Results are given in table 6.2. It can be seen that there are significantly increased odds ratios (OR) of a number of disease in both the MS and the sibling population. These are mainly autoimmune diseases: Hashimotos disease, pernicious anaemia, celiac disease and inflammatory bowel disease all appear to be more common in both the MS and the sibling populations. This highlights the immunopathic trait that forms part of the endophenotype.

Table 6.2: Calculated odds ratios (OR) of selected diseases in the MS and sibling study population

Disease	OR in all MS (95% CI)	OR in female MS (95% CI)	OR in male MS (95% CI)	OR in all siblings	OR in female siblings (95% CI)	OR in male siblings (95% CI)
Hashimotos/hypothyroidism	N/A	2.52 (0.82-7.70)	**62.5 (9.49-411.6)**	N/A	**6.38 (3.38-12.03)**	13.16 (1.81-95.58)
Type 1 diabetes	No cases	No cases	No cases	2.43 (0.34-17.61)	3.54 (0.49-25.58)	No cases
Type 2 diabetes	N/A	0.42 (0.06-3.02)	No cases	N/A	1.06 (0.33-3.39)	No cases
Rheumatoid arthritis	No cases	No cases	No cases	N/A	1.13 (0.16-7.97)	No cases
Pernicious anaemia	**19.72 (4.53-85.95)**	**21.98 (5.05-95.59)**	No cases	**12.71 (2.90-55.74)**	**18.54 (4.25-80.85)**	No cases
Systemic lupus erythematosus (SLE)	N/A	No cases	No cases	N/A	**50.20 (12.40-203.17)**	No cases
Psoriasis	No cases	No cases	No cases	1.10 (0.26-4.76)	0.80 (0.11-6.01)	1.75 (0.24-12.94)
Coeliac disease	N/A	**26.46 (3.71-188.58)**	No cases	N/A	**22.31 (3.12-159.40)**	No cases
Inflammatory bowel disease	**9.86 (2.38-40.84)**	**10.99 (2.66-45.41)**	No cases	**6.36 (1.53-26.49)**	**9.27 (2.24-38.42)**	**20.24 (4.98-82.29)**
Seizures	1.71 (0.24-12.14)			1.10 (0.15-7.86)		

N/A: unable to compare overall population rates to overall rates in this cohort due to differential rates in males and females

6.1.4. Overall risk of comorbid autoimmune disease in MS

As discussed above, there is therefore a large amount of information available examining the frequency of autoimmune disease both in people with MS and their first-degree relatives. I set out to perform a systematic review of the frequency of selected autoimmune diseases (autoimmune thyroid disease, type 1 diabetes mellitus, inflammatory bowel disease, psoriasis, systemic lupus erythematosus and rheumatoid arthritis) in both people with MS and their first-degree relatives. Heterogeneity between studies was assessed, and, where possible, overall estimates for the frequency of these diseases in both people with MS and their first-degree relatives calculated.

6.1.4.1. Inclusion criteria

Inclusion criteria were pre-specified. Papers selected for inclusion were those published after 1980 which gave figures for the prevalence of specified autoimmune diseases in both MS and healthy control populations. The control population had to be matched to the MS population in terms of age and sex, or alternatively a precise local population prevalence of autoimmune disease had to be given (approximations of overall population rates were not felt to be sufficiently precise). The control population for the "relatives of MS" population could be either directly matched, or alternatively the probands matched and their families compared.

6.1.4.2. Search strategy

PubMed and Web of Science were searched using the terms “multiple sclerosis” AND “thyroid”, “multiple sclerosis” AND “diabetes”, “multiple sclerosis” AND “Crohn’s”, “multiple sclerosis” AND “Crohns” “multiple sclerosis” AND “ulcerative colitis”, “multiple sclerosis” AND “inflammatory bowel disease”, “multiple sclerosis” AND “psoriasis”, “multiple sclerosis” AND “lupus”, “multiple sclerosis” AND “SLE”, “multiple sclerosis” AND “rheumatoid” and “multiple sclerosis” AND “arthritis”. The resulting abstracts were hand-searched for publications meeting the inclusion criteria. The results from each search were cross-referenced, as many of the included papers examined more than one autoimmune disease.

6.1.4.3. Statistical analysis

A generic inverse variance fixed or random effects model was used for the statistical analysis as appropriate. A random effects model was applied unless I^2 was ≤ 25%; in which case a fixed effects model was used (15). Between-study heterogeneity was assessed for each calculation using Cochran's Q chi-square test and I^2 (16). Where present, heterogeneity was explored using subgroup analysis. Risks were reported as pooled OR and 95% confidence intervals (CI). Bias was assessed using visual inspection of funnel plots; and where >10 studies were included, quantified using an Egger p-value (17). A p-value of <0.05 was considered statistically significant. Analyses were conducted using RevMan 5.1 (Cochrane collaboration).

6.1.4.4. Included studies

Following the initial searches 254 unique papers and 4 conference abstracts were assessed in order to ascertain whether the inclusion criteria were met. All 4 conference abstracts were rejected, as the same cohorts were used in later published articles. 41 unique papers were initially selected for inclusion, and the selection process is summarised in figure 6.1. Two studies were later excluded from the analysis as the number of relatives was not given, only the number of index MS cases (304, 305). The remaining 39 papers, details of which are given in table 6.3, were used in the analysis.

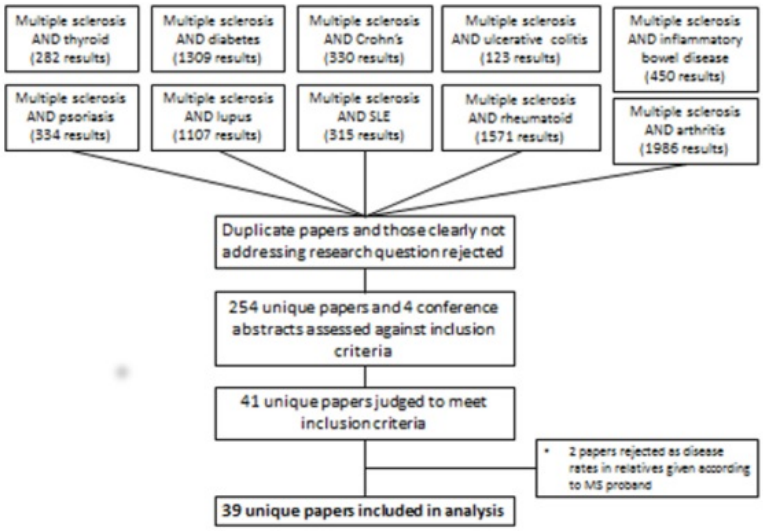


Figure 6.1: Study selection

Table 6.3: Included studies

Study	Data collection method and study type	Autoimmune disease(s)	Control population	Number cases/controls/ relatives/control relatives	Comorbidity (n(%))			
					Cases	Controls	Relatives	Control relatives
Annunziata 1999 (306)	Direct measurement Case-control	Thyroid autoantibodies (TPO and Tg)	OND and HC	129/282	28 (21.7) (TPO) 11 (8.5) (Tg)	20 (7.1) 11 (3.9)		
Broadley 2000 (291)	Questionnaire Case-control	AIT	Matched families	571/375/ 2124/1315			263 (12.4)	71 (3.3)
		SLE					13 (0.6)	0 (0)
		T1DM					47 (2.2)	18 (1.4)
		Psoriasis					195 (9.2)	87 (6.6)
		IBD					32 (1.5)	28 (2.1)
		RA					47 (2.2)	29 (2.2)
Dallmeijer 2009 (307)	Clinical review Cohort	DM	Stroke patients	146/198	6 (4.1)	23 (11.6)		
De Keyser 1988 (308)	Notes review Cross sectional	Hypothyroidism	OND	828/100	4 (0.5)	1 (1.0)		
		T1DM			4 (0.5)	1 (1.0)		
		UC			2 (0.2)	0 (0)		
		RA			5 (0.6)	1 (1.0)		
	Direct measurement Case-control	Thyroid autoantibodies		105/105	4 (4.0)	2 (2.0)		
		ANA			20 (19.0)	8 (7.6)		
Deretzi 2010 (309)	Questionnaire Case-control	AIT	HC	891/355/ 3112/1580			258 (8.3)	52 (3.3)
		SLE					20 (0.6)	9 (0.6)
		IDDM					37 (1.2)	6 (0.4)
		Psoriasis					33 (1.1)	9 (0.6)
		IBD					60 (1.9)	9 (0.6)
		RA					7 (0.2)	4 (0.3)

Dore-Duffy 1982 (310)	Direct measurement Case-control	ANA	HC	27/20	22 (81.4)	4 (20.0)		
Durelli 2001 (311)	Direct measurement Case-control	Thyroid autoantibodies (TMA)	Blood donors	156/437	9 (5.8)	16 (3.7)		
Edwards 2004 (312)	Questionnaire Cross-sectional	AIT	Local population	658/2779	21 (3.2)	50 (1.8)		
		T1DM		658/252538	6 (0.9)	128 (0.1)		
		Psoriasis			9 (1.4)	1836 (0.7)		
		RA			2 (0.3)	824 (0.3)		
		UC		658/136000	5 (0.8)	330 (0.2)		
		Crohn's			2 (0.3)	196 (0.1)		
Henderson 2000 (313)	Questionnaire Case-control	Clinical thyroid dysfunction	HC	117/222/ 722/1582	6 (5.1)	15 (6.8)	12 (1.7)	11 (0.7)
		Hypothyroidism			3 (2.6)	8 (3.6)		
		SLE			2 (1.7)	1 (0.5)	6 (0.8)	7 (0.4)
		T1DM			1 (0.9)	2 (1.0)	5 (0.7)	4 (0.3)
		Psoriasis			6 (5.1)	7 (3.2)	9 (1.2)	17 (1.1)
		IBD			3 (2.6)	0 (0)	2 (0.3)	4 (0.3)
		RA			4 (3.4)	1 (0.5)	11 (1.5)	14 (0.9)
Hyypia 1982 (314)	Direct measurement Case-control	Anti-dsDNA antibodies	HC	30/30	3 (10)	3 (10)		
Ioppoli 1990 (315)	Direct measurement Case-control	Thyroid autoantibodies (Tg and TMA)	OND	113/51	19 (16.8)	3 (5.9)		
Iwasaki 1988 (316)	Direct measurement Case-control	Clinical thyroid dysfunction	Non-neurological patients	48/50	0 (0)	0 (0)		
		Thyroid autoantibodies (Tg and TMA)			0 (0)	0 (0)		
Kang 2010	Database	Hypothyroidism	HC	898/4490	15 (1.7)	24 (0.5)		

(288)	Case-control	Hypothyroidism SLE	HC	898/4490	15 (1.7) 26 (2.9)	24 (0.5) 5 (0.1)		
		T1DM RA			3 (0.3) 29 (3.2)	1 (0.0) 31 (0.7)		
Karni 1999 (317)	Direct measurement Prospective controlled	Clinical thyroid dysfunction	OND	391/158	31 (7.9) (all thyroid dysfunction) 25 (6.4) (hypothyroid)	5 (3.2) (all thyroid dysfunction 4 (2.5) (hypothyroid)		
Langer-Gould 2010 (287)	Database Case-control	Hashimoto's disease SLE	HC	5296/26478	9 (0.2) 20 (0.4)	47 (0.2) 75 (0.3)		
		T1DM Psoriasis			45 (0.8) 70 (1.3)	240 (0.9) 319 (1.2)		
		IBD RA			42 (0.8) 44 (0.8)	120 (0.5) 228 (0.9)		
Laroni 2006 (318)	Questionnaire Case-control	AIT DM prior to age 20	HC	245/245/ 984/1002	9 (3.7) 9 (3.7)	7 (2.9) 1 (0.4)	13 (1.3) 8 (0.8)	10 (1.0) 3 (0.3)
		Psoriasis RA			1 (0.4) 2 (0.8)	2 (0.8) 2 (0.8)		
Lindegard 1985 (319)	Prospective collection Population-based cohort	DM	Epilepsy patients	351/548	17 (4.8)	33 (6.0)		
Marrosu 2002 (320)	Notes review Cross-sectional	T1DM	HC	1090/35906/ 5480/35906	28 (2.6)	194 (0.5)	53 (1.0)	194 (0.5)
Midgard 1996 (321)	Questionnaire Case-control	Goitre DM	Hospital inpatients	155/200/717/991	4 (2.6) 0 (0)	1 (0.5) 0 (0)	12 (1.7) 25 (3.5)	12 (1.2) 21 (2.1)

		Psoriasis RA			12 (7.7) 3 (1.9)	8 (4.0) 1 (0.5)	12 (1.7)	23 (2.3)
Michielsens 1991 (322)	Direct measurement Case-control	ANA	OND	48/327	1 (2.1)	30 (9.2)		
Munteis 2007 (323)	Direct measurement Case-control	Clinical thyroid dysfunction	HC	93/401	0 (0)	0 (0)		
	Direct measurement Case-control	Thyroid autoantibodies (Tg and TPO)			11 (11.8)	10 (2.5)		
Niederwieser 2003 (324)	Clinical review Case-control	AIT	OND	353/308	84 (24.7)	64 (20.8)		
	Direct measurement Case-control	Thyroid autoantibodies			31 (8.8)	21 (6.8)		
Nielsen 2006 (284)	Database Population-based cohort	DM prior to age 20	HC	x/x/14771/14771			56 (0.4)	39 (0.3)
Nielsen 2008 (285)	Database Population-based cohort	Hashimoto	HC	10596/10596/ 20800/20800	0 (0)	0 (0)	0 (0)	0 (0)
		SLE			2 (0.0)	4 (0.0)	9 (0.1)	7 (0.1)
		Psoriasis UC			18 (0.2) 29 (0.3)	12 (0.1) 15 (0. 1)	16 (0.1) 51 (0.2)	22 (0.1) 39 (0.2)
		Crohn's RA			6 (0.1) 28 (0.3)	9 (0.1) 53 (0.5)	44 (0.2) 57 (0.3)	31 (0.1) 49 (0.2)
Pokorny 2007 (325)	Medical notes review Cross-sectional	UC	HC	496/100000	4 (0.8)	80 (0.1)		
Ramagopalan 2007 (326)	Questionnaire Population-based cohort	AIT	Spouses of MS index cases	5031/2707/ 30259/2707	395 (7.8)	116 (4.3)		
		SLE			28 (0.6)	7 (0.3)		

		T1DM			19 (0.4)	14 (0.5)	112 (0.4)	14 (0.5)
		Psoriasis			293 (5.8)	146 (5.4)		
		UC			9 (0.2)	4 (0.1)	88 (0.3)	4 (0.1)
		Crohn's			11 (0.2)	4 (0.1)	57 (0.2)	4 (0.1)
		RA			153 (3.0)	66 (2.4)	529 (1.7)	66 (2.4)
Roshanisefat 2012 (283)	Database Population-based cohort	T1DM	HC	20276/203951/ 23242/251423	966 (4.8)	8611 (4.2)	1730 (7.4)	18558 (7.4)
		Psoriasis			122 (0.6)	800 (0.3)	119 (0.5)	1126 (0.4)
		UC			113 (0.6)	819 (0.4)	82 (0.4)	821 (0.3)
		Crohn's			93 (0.5)	669 (0.3)	58 (0.2)	609 (0.2)
		RA			159 (0.8)	2130 (1.0)	369 (1.6)	3849 (1.5)
Seyfert 1990 (327)	Questionnaire Case-control	Graves' disease and hypothyroidism	OND	101/97	6 (5.9)	2 (2.1)		
	Direct measurement Case-control	Thyroid autoantibodies (TMA, Tg and TSH-r)		88/95	10 (11.4)	5 (5.3)		
Sloka 2005 (328)	Medical notes review Population-based cohort	AIT	OND	491/532	41 (8.4)	14 (2.6)		
Somers 2009 (286)	Database Population-based cohort	Hypothyroidism	HC	4332/4332	61 (1.4)	42 (1.0)		
		IDDM			175 (4.0)	39 (0.9)		
		RA			30 (0.7)	38 (0.9)		
Spadaro 1999 (329)	Direct measurement Case-control	Clinical thyroid dysfunction	Blood donors	105/75	0 (0)	0 (0)		
		Thyroid autoantibodies (TMA, TPO and Tg)			18 (17.1) (TMA/TPO)	2 (2.7)		
					16 (15.2) (Tg)	1 (1.3)		

		ANA			35 (33.3)	2 (2.7)		
Szmyrka-Kaczmarek 2012 (330)	Direct measurement Case control	ANA and dsDNA antibodies	HC	85/30	54 (63.5) (ANA)	1 (3.3)		
					3 (12.8) (dsDNA)	0 (0)		
Warren 1981 (44)	Questionnaire Case-control	DM	Neurology and rheumatology inpatients	100/100/1088/ 1146	3 (3)	0 (0)	42 (3.9)	29 (2.5)
Warren 1982 (45)	Questionnaire Case-control	DM	HC	100/100/1996/ 1851	8 (8)	6 (6)	30 (1.5)	17 (0.9)
Wei 1997 (331)	Direct measurement Case control	Clinical thyroid dysfunction	OND and HC	52/15	0 (0)	0 (0)		
Wei 2010 (332)	Direct measurement Case-control	Thyroid autoantibodies (TPO)	HC	149/92	15 (10.1)	16 (17.4)		
Wertman 1992 (333)	Database Population-based cohort	T1DM	HC	334/334	3 (1.0)	0 (0)		
Wynn 1990 (334)	Database Population-based cohort	AIT T1DM	HC	191/191	3 (1.6) 9 (4.7)	2 (1.0) 6 (3.1)		

OND: Other non-inflammatory neurological diseases
AIT: all autoimmune thyroid disease
T1DM: type 1 diabetes mellitus
UC: ulcerative colitis
RA: rheumatoid arthritis
TPO: thyroid peroxidase antibodies
TSH-r: TSH-receptor antibodies
ANA: antinuclear antibodies

HC: healthy controls, without evidence of MS
SLE: systemic lupus erythematosus
DM: diabetes mellitus
IBD: inflammatory bowel disease
TMA: thyroid microsomal antibodies
Tg: anti-thyroglobulin antibodies
dsDNA: anti-double stranded DNA antibodies

6.1.4.5. Rates of autoimmune disease

Overall results are given in table 6.3, and discussed in more detail below.

6.1.4.5.1. Thyroid autoimmunity

6.1.4.5.1.1. Clinical thyroid dysfunction

The majority of hypothyroidism in iodine-replete areas is autoimmune in nature (289), but the same cannot be said of hyperthyroidism. Some studies therefore used hypothyroidism as a surrogate diagnosis for autoimmune thyroid dysfunction. Nineteen studies examined thyroid function in MS (285-288, 308, 312, 313, 316-318, 321, 323, 324, 326-329, 331, 334). In five of these studies (285, 316, 323, 329, 331) there were no cases of thyroid dysfunction in either MS patients or controls, therefore these could not be included in the analysis. The remaining fourteen studies gave an overall increased risk of thyroid dysfunction in people with MS (OR 1.66, 95%CI 1.35-2.05, p<0.00001), without between-study heterogeneity (Cochran's Q p=0.16, I^2=27%) (figure 6.2a). A funnel plot demonstrated no significant publication bias (figure 6.3a), with an Egger p-value of 0.76. When those studies using hypothyroidism as a marker of autoimmune thyroid disease were selected (286, 288, 308, 313, 317), there was an increased risk in people with MS (OR 1.72, 95%CI 1.00-2.97, p=0.05) with no heterogeneity. A similar effect was seen when only those studies specifying “autoimmune thyroid disease” were selected (312, 318, 324, 326, 328, 334) (OR 1.72, 95%CI 1.46-2.04, p<0.00001). When cases of Hashimoto’s thyroiditis were analysed separately (287, 318, 328) there was no increased risk in people with MS (OR 1.42, 95%CI 0.72-2.79, p=0.31).

When looking at thyroid function in first degree relatives of people with MS, seven studies were identified (285, 291, 305, 309, 313, 318, 321). One study was excluded (305) as only the number of MS index cases was given, rather than the number of relatives, and one included no cases of thyroid dysfunction (285). There was an overall increased risk of thyroid dysfunction in first-degree relatives

of people with MS (OR 2.38, 95%CI 1.95-2.91, p<0.00001) (figure 6.1b) with very few points on a funnel plot leading to difficulty in assessing bias (figure 6.2b).

Table 6.3: Calculated OR for each autoimmune disease in both MS patients and their first degree relatives.

Disease		OR in MS (OR; 95% CI)	Heterogeneity	Egger p-value (bias)	OR in first degree relatives of MS (OR; 95% CI)	Heterogeneity	Egger p-value (bias)
Thyroid autoimmunity	Clinical thyroid dysfunction	**1.66; 1.35-2.05 p<0.00001**	X^2=17.88, df=13 (p=0.16) I^2=27%	0.76	**2.38; 1.95-2.91 p<0.00001**	X^2=4.26, df=4 (p=0.37) I^2=6%	N/A
	Thyroid autoantibodies	**2.36; 1.32-4.20 p=0.004**	**X^2=31.04, df=8 (p=0.0001) I^2=74%**	0.56	N/A		
Type 1 diabetes mellitus	All studies	**2.02; 1.22-3.40 p=0.006**	**X^2=174.89, df=15 (p<0.00001) I^2=91%**	0.16	**1.49; 1.15-1.94 p=0.002**	**X^2=38.12, df=10 (p<0.0001) I^2=74%**	**0.003**
	Conservative analysis	**2.69; 1.43-5.04 p=0.002**	**X^2=162.08, df=10 (p<0.00001) I^2=94%**	N/A			
Inflammatory bowel disease	Crohn's disease	**1.37; 1.12-1.37 p=0.003**	X^2=2.29, df=3 (p=0.51) I^2=0%	N/A	1.13; 0.90-1.41 p=0.31	X^2=1.45, df=2 (p=0.48) I^2=0%	N/A
	Ulcerative colitis	**2.26 1.23-4.14 p=0.009**	**X^2=17.99, df=5 (p=0.003) I^2=72%**	N/A	1.15; 0.95-1.40 p=0.15	X^2=1.76, df=2 (p=0.41) I^2=0%	N/A
	Overall	**1.56; 1.28-1.90 p<0.0001**	X^2=6.38, df=5 (p=0.27) I^2=22%	N/A	1.29; 0.92-1.82 p=0.14	**X^2=15.71, df=5 (p=0.008) I^2=68%**	N/A
Psoriasis		**1.31; 1.09-1.57 p<0.0001**	X^2=10.63, df=7 (p=0.16) I^2=34%	N/A	1.17; 0.94-1.46 p=0.16	X^2=7.68, df=5 (p=0.17) I^2=35%	N/A
Systemic Lupus Erythematosus		2.80; 0.76-10.25 p=0.12	**X^2=33.06, df=4 (p<0.00001) I^2=88%**	N/A	1.53; 0.87-2.69 p=0.14	X^2=3.39, df=3 (p=0.33) I^2=12%	N/A
Rheumatoid arthritis		1.15; 0.77-1.73 p=0.49	**X^2=62.11, df=10 (p<0.00001) I^2=84%**	0.28	0.98; 0.80-1.20 p= 0.87	X^2=9.54, df=5 (p=0.09) I^2=48%	N/A

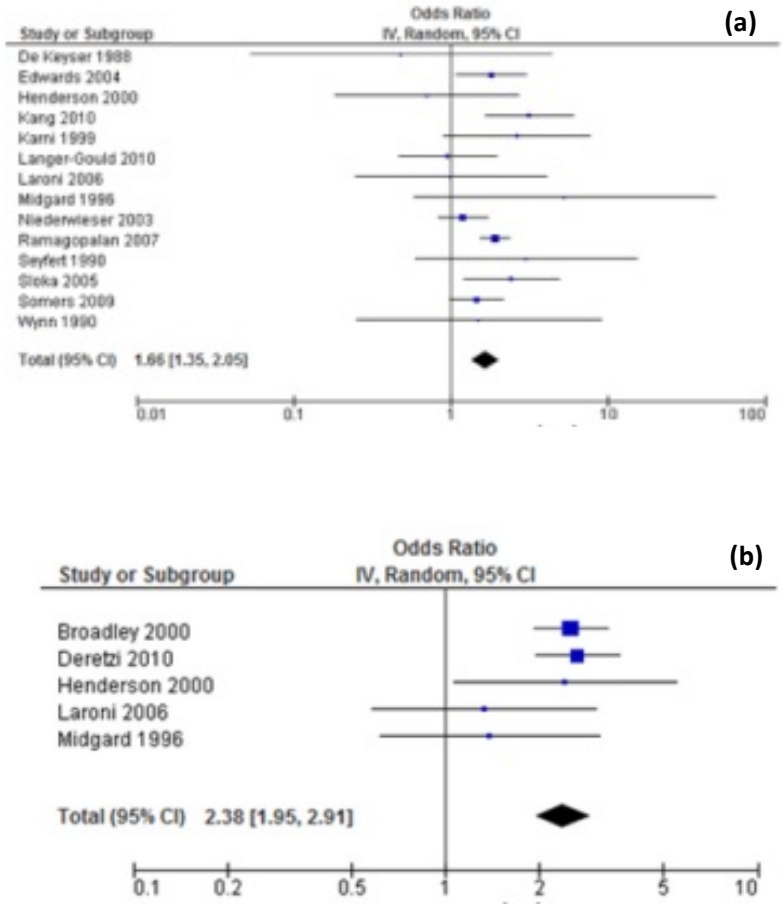


Figure 6.2: (a) Forest plot demonstrating the OR of clinical thyroid disease in people with MS. **(b)** Forest plot demonstrating the OR of clinical thyroid disease in relatives of people with MS.

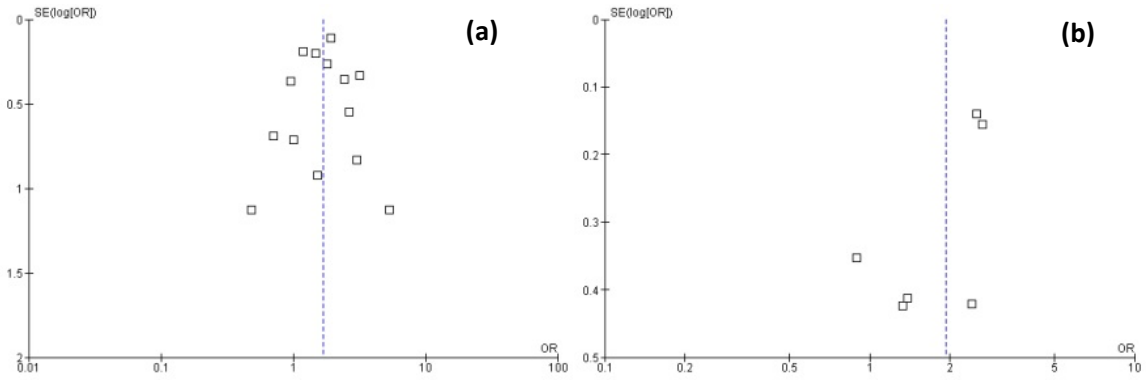


Figure 6.3: (a) Funnel plot demonstrating the lack of publication bias when examining the frequency of thyroid disease in people with MS. **(b)** Funnel plot demonstrating the difficulty examining publication bias given the small number of datapoints.

6.1.4.5.1.2. Thyroid autoantibodies

Ten studies examined thyroid autoantibodies in MS (306, 308, 311, 315, 316, 323, 324, 327, 329, 332). One study (316) did not detect any antibodies in either MS patients or controls, and was excluded from the analysis. There was an overall increased rate of thyroid autoantibodies in patients with MS compared to healthy controls (OR 2.36, 95%CI 1.32-4.20, p=0.004) but with significant heterogeneity (Cochran's Q p=0.0001, I^2=74%). There was no evidence of publication bias (Egger p-value=0.56). Heterogeneity was explored by examining each thyroid autoantibody individually, however each attempt at subgroup analysis resulted in a small number of studies being examined. No studies gave data regarding the rate of thyroid autoantibodies in relatives of MS patients compared to healthy controls.

6.1.4.5.2. Type 1 diabetes mellitus

Seventeen studies (283, 286-288, 307, 308, 312, 313, 318-321, 326, 333-336) gave data regarding the number of people with MS and co-existing diabetes. One study (321) contained no cases of diabetes in either people with MS or controls. There was an increased risk of diabetes associated with MS overall (OR 2.02, 95%CI 1.22-3.40, p=0.006) (figure 6.4a). However, this was associated with significant heterogeneity when all studies were considered together (Cochran's Q p<0.00001, I^2=91%). There was no evidence of publication bias (Egger p-value=0.16) (figure 6.5a). The potential reasons for the heterogeneity seen were explored by only including those studies that specified type 1 diabetes or insulin dependent diabetes (excluded studies: (319, 334-336)). A study using patients with stroke as the control group was also excluded (307), given the association of stroke with diabetes. This strengthened the relationship between MS and diabetes (OR 2.69, 95%CI 1.43-5.04), but heterogeneity remained (Cochran's Q p<0.00001, I^2=94%). Separating studies using large databases from those using questionnaires did not affect heterogeneity. Funnel plots of the

subgroup analyses did not reveal any evidence of publication bias, but supported the high degree of heterogeneity found.

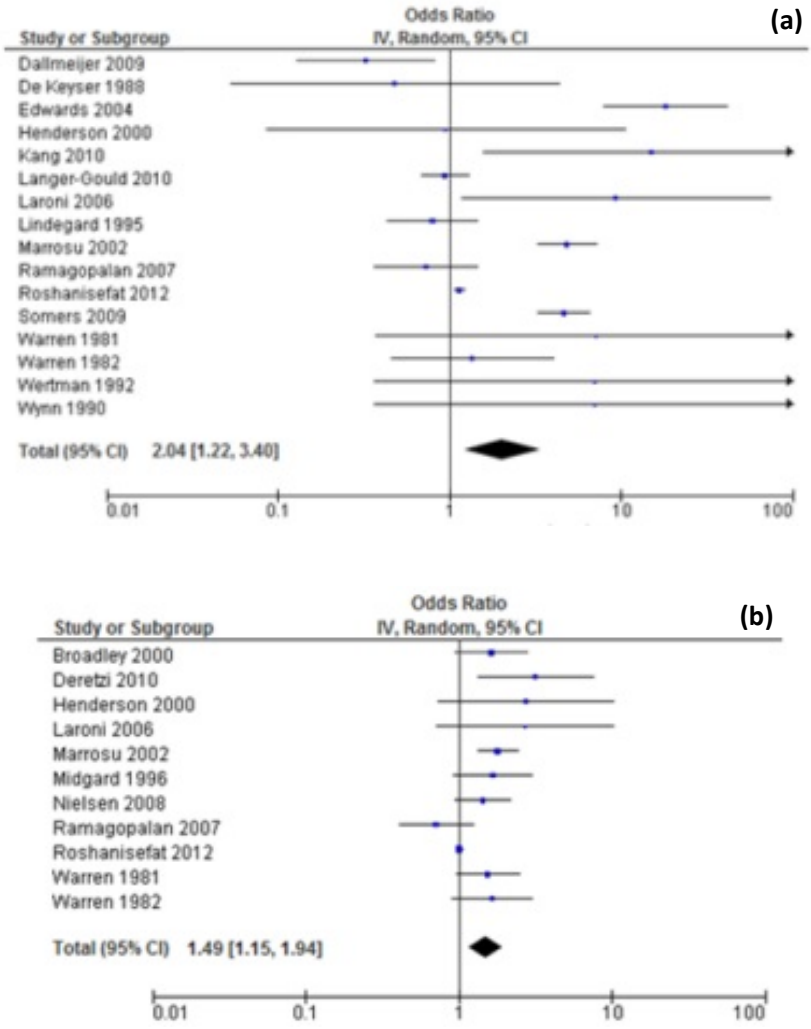


Figure 6.4: (a) Forest plot demonstrating the OR of diabetes in people with MS. **(b)** Forest plot demonstrating the OR of diabetes in relatives of people with MS.

Eleven studies examined the risk of diabetes in first-degree relatives of people with MS (283, 284, 291, 309, 313, 318, 320, 321, 326, 335, 336). There was an overall increased risk of diabetes in relatives of people with MS (OR 1.49, 95%CI 1.15-1.94, p=0.002) (figure 6.4b). This result was associated with significant heterogeneity (Cochran's Q p<0.0001, I^2=74%), and there was evidence of publication bias when the data was examined using a funnel plot (Egger p-value=0.003) (figure 6.5b),

with the smaller studies showing a greater effect size. When the two studies not specifying T1DM were excluded (335, 336), a similar result was obtained (OR 1.48, 95%CI 1.10-2.00, p=0.01). Only two studies (283, 284) used databases to examine the OR of type 1 diabetes in first-degree relatives of people with MS. When these studies were examined separately no increase in diabetes risk was seen (OR 1.13, 95%CI 0.82-1.57), with no significant heterogeneity and insufficient studies to assess publication bias (figure 6.5c). Interestingly, when those studies using a questionnaire design were used (291, 309, 313, 318, 320, 321, 326), there did appear to be an increase in the risk of type 1 diabetes in first-degree relatives of people with MS (OR 1.65, 95%CI 1.17-2.35, p=0.005) with no significant heterogeneity between studies. However, a funnel plot revealed evidence of publication bias amongst these studies (figure 6.5d), severely limiting the applicability of the results.

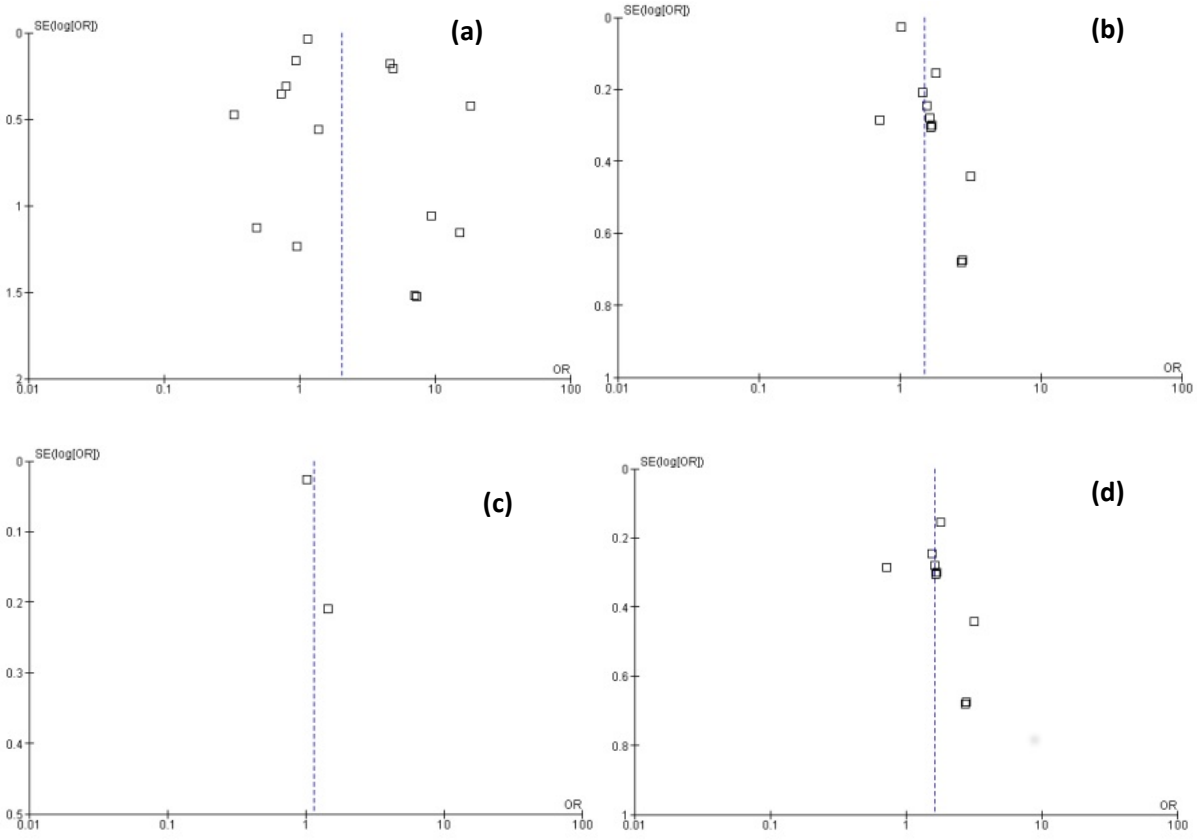


Figure 6.5: (a) Funnel plot demonstrating lack of publication bias in frequency of diabetes in people with MS. **(b)** Publication bias when all studies in relatives of people with MS are included. **(c)** Insufficient studies to assess publication bias in type 1 diabetes in relatives of people with MS. **(d)** Publication bias present when questionnaire studies are selected.

6.1.4.5.3. Inflammatory bowel disease

6.1.4.5.3.1. Crohn's disease

Four studies (283, 285, 312, 326) examined the number of people with MS and Crohn's disease. There was a significantly increased risk of Crohn's disease in people with MS (OR 1.37, 95% CI 1.12-1.69, p=0.003). Three studies (283, 285, 326) examined the risk of Crohn's in first-degree relatives of people with MS and no increased risk was found (OR=1.13, 95% CI 0.90-1.41, p=0.31). There was no significant heterogeneity, and publication bias (examined using funnel plots) did not appear to be present in either analysis.

6.1.4.5.3.2. Ulcerative colitis (UC)

Six studies examined the number of people with MS who also had UC (283, 285, 308, 312, 325, 326). Again, there was an increased risk of UC in people with MS (OR 2.26, 95% CI 1.23-4.14, p=0.009), however with significant heterogeneity (Cochran's Q p=0.003, I^2=72%). Three studies (283, 285, 326) examined the risk of UC in relatives of people with MS; no increase was found (OR 1.15, 95% CI 0.95-1.40, p=0.15). There was no publication bias present on funnel plot.

6.1.4.5.3.3. All inflammatory bowel disease

Using the information from six studies (283, 285, 287, 312, 313, 326) the overall OR associated with MS for inflammatory bowel disease was calculated. This showed an increased risk of inflammatory bowel disease with MS (OR 1.56, 95% CI 1.28-1.90, p<0.0001) (figure 6.6a). No increase in risk was seen in relatives of people with MS (OR 1.29, 95% CI 0.92-1.82, p=0.14) (283, 285, 291, 309, 313, 326) (figure 6.6b). There was no significant publication bias seen on funnel plot (figures 6.7a and 6.7b), although heterogeneity was observed between those studies examining relatives (Cochran's Q p=0.008, I^2=68%).

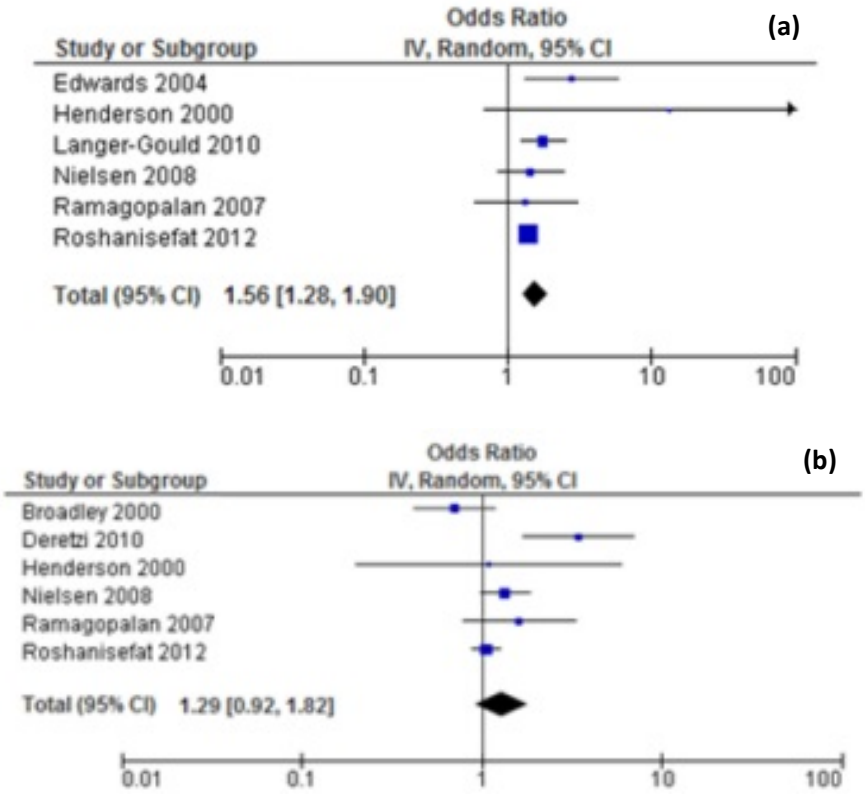


Figure 6.6: (a) Forest plot demonstrating the OR of inflammatory bowel disease in people with MS. **(b)** Forest plot demonstrating the OR of inflammatory bowel disease in relatives of people with MS.

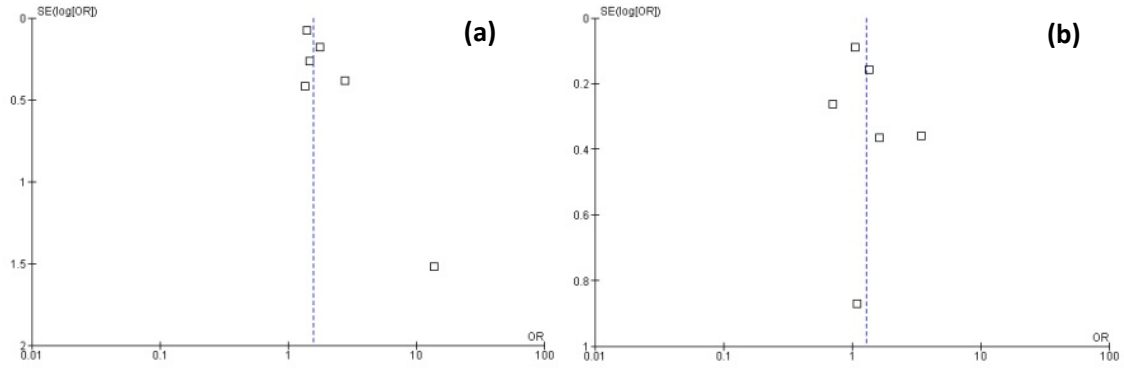


Figure 6.7: (a) Funnel plot demonstrating the lack of publication bias when examining the frequency of inflammatory bowel disease in people with MS. **(b)** Funnel plot demonstrating the lack of publication bias when examining the frequency of inflammatory bowel disease in relatives of people with MS.

6.1.4.5.4. Psoriasis

Eight studies examined the risk of psoriasis in people with MS (283, 285, 287, 312, 313, 318, 321, 326) (figure 6.8). There was a significant increase in the risk of psoriasis in people with MS (OR 1.31, 95%CI 1.09-1.57, p<0.0001). There was no significant between-study heterogeneity (Cochran's Q p=0.16, I^2=34%). Six studies examined the risk of psoriasis in first-degree relatives of people with MS (283, 285, 291, 309, 313, 321). There was no increased risk of psoriasis in first-degree relatives of people with MS (OR 1.17, 95%CI 0.94-1.46, p=0.16) and no heterogeneity or publication bias was detected (figures 6.9a and 6.9b).

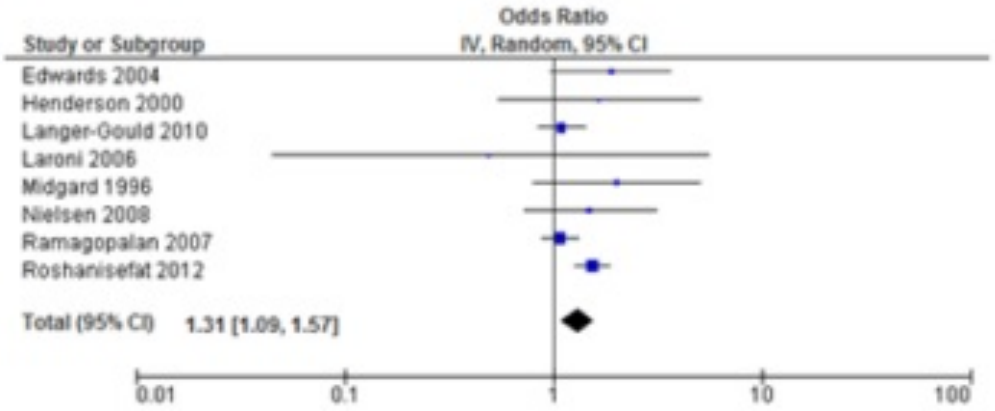


Figure 6.8: Forest plot demonstrating the OR of psoriasis in people with MS.

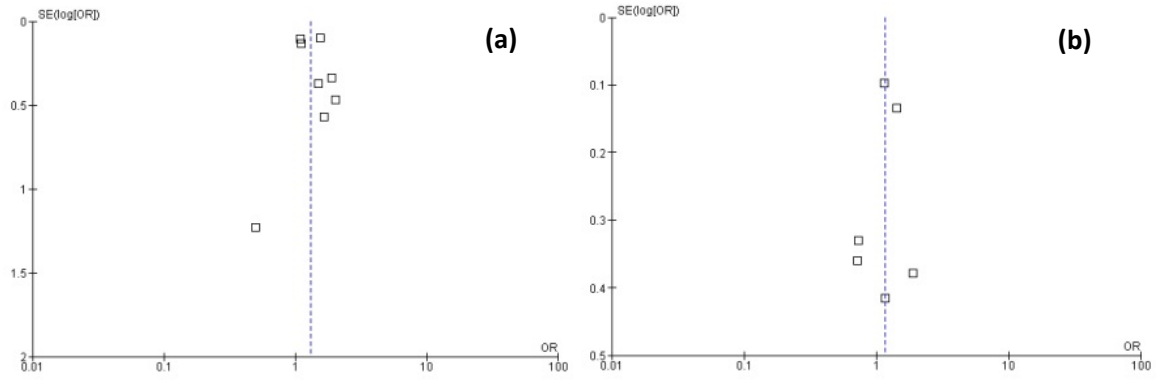


Figure 6.9: (a) Funnel plot demonstrating the lack of publication bias when examining the frequency of psoriasis in people with MS. **(b)** Funnel plot demonstrating the lack of publication bias when examining the frequency of psoriasis in relatives of people with MS.

6.1.4.5.5. Systemic lupus erythematosus (SLE)

Studies examining the risk of SLE in MS took one of two forms, using either clinical diagnosis or serology (i.e. the presence of autoantibodies). Five studies used clinical diagnosis as the measure of interest (285, 287, 288, 313, 326). There did not appear to be an increased risk of SLE in those with MS (OR 2.80, 95%CI 0.76-10.25, p=0.12) although heterogeneity was high (Cochran's Q p<0.00001, I^2=88%). There appeared to be an increased risk of detectable ANA (308, 310, 322, 329, 330) (OR 6.36, 95%CI 1.36-29.69), but with a high 95%CI and heterogeneity. There was no increased risk of detectable dsDNA antibodies (314, 330) (OR 1.26, 95%CI 0.29-5.47) in MS. No significant publication bias was seen in either analysis using a funnel plot.

All studies examining the risk of SLE in first-degree relatives of people with MS (285, 291, 309, 313) used a clinical diagnosis of SLE. There was no increase in risk of SLE (OR 1.53, 95%CI 0.87-2.69).

6.1.4.5.6. Rheumatoid arthritis (RA)

Eleven studies examined the risk of RA in MS (283, 285-288, 308, 312, 313, 318, 321, 326). There was no association between MS and RA seen in either MS patients (OR 1.15, 95%CI 0.77-1.73, p=0.49) or relatives (OR 0.98, 95% CI 0.80-1.20, p=0.87). There was significant heterogeneity between studies examining MS cases, but no publication bias was demonstrated (Egger p-value=0.28). When studies using a questionnaire design were selected (312, 313, 318, 321, 326), the lack of association between MS and RA persisted (OR 1.29, 95% CI 0.98-1.71, p=0.07) but without heterogeneity (Cochran's Q p=0.43, I^2=0%).

6.1.4.6. Conclusions

It can therefore be seen that there is an immunopathic trait not only in people with MS but also in their siblings. This is seen both in the siblings recruited as part of this endophenotype study, but also in a wider study encompassing all of the published literature.

On a population level, the only autoimmune diseases showing an increased rate in relatives of people with MS are thyroid disease and type 1 diabetes. Thyroid disease is relatively common in the general population. The symptoms of thyroid disease tend to be non-specific and progress insidiously. The finding that there is a consistent increase in the rate of thyroid disease both in the MS population and in their relatives should prompt the consideration of baseline testing of thyroid function in people with MS, and alert clinicians to consider thyroid dysfunction in those patients reporting non-specific symptoms who have not had thyroid function checked recently. The increase in the rate of thyroid autoantibodies, although of interest, should not prompt screening for these in the MS population. Thyroid autoantibodies may be present in healthy people with normal thyroid function, with the prevalence of thyroid peroxidase antibodies reported to be as high as 12% in some series of healthy individuals (337).

The absolute numbers in the sibling study are relatively small. It is possible that selection and/or reporting bias influenced the results obtained in this section of the study, as those siblings of people with MS who have additional autoimmune disease may be more likely to enter a study looking at the genetic causes of disease, and recall bias may also play a role. It has been argued that the increased rate of autoimmune disease in MS is seen only after the diagnosis of MS is made, and this is thought to be as a result of the increased contact that people with MS have with healthcare professionals. A similar feature may be at play in the siblings of people with MS – having experienced the diagnosis of their brother or sister they may be more likely to report vague or chronic symptoms.

When thinking about the findings of the meta-analysis, it is important to bear in mind that the accuracy of the results are limited by the quality of the studies informing it. Despite the best efforts of the authors of the studies included here, it is highly likely that diseases are misclassified within the studies included here. When comparing self-report to GP verified diagnosis, Broadley et al (291) found that the positive predictive value of a patient reported-condition varied from 32% for rheumatoid arthritis to 85% for thyroid disease (291). This is a major limitation for questionnaire-based studies. Similarly, reporting bias may have led to over-estimation of autoimmune disease prevalence amongst people with MS and their relatives. This is particularly apparent in those studies examining the frequency of diabetes in relatives of people with MS, where there is clear evidence of publication bias. However, the majority of the more recent studies use large-scale databases, potentially minimising these sources of bias. Interestingly, in the case of diabetes in relatives, the effect of MS disappears when studies using databases are analysed in isolation, highlighting the benefits of such studies.

This work does not address the potential cause(s) of the increased rate of autoimmune diseases demonstrated in both people with MS and their siblings. This is likely to be multifactorial, as the diseases studied have differing underlying aetiologies and pathogenesis. Common factors in the development of MS and these diseases include both genetic and environmental factors, including smoking and vitamin D deficiency. The conditions studied do not have a single underlying pathogenesis, and as such it is difficult to use this study to shed light on the mechanisms underlying MS development. It does however point to the possibility of an underlying endophenotypic trait, with increased susceptibility to other autoimmune diseases.

6.2. Serological and urinary biomarkers

6.2.1. Urinary free light chains

One aim of this study was to validate the MS risk score generated for siblings of people with MS using serological and urinary markers known to be increased in people with MS. One of the markers selected for further study was urinary free light chains (FLC). FLC are known to be increased in the CSF of people with MS, and their presence is strongly associated with OCBs (see chapter 3). They have also been detected at increased concentrations in the urine of people with MS (41, 43, 192).

A hypothesis of this study was that some siblings of people with MS would also have increased FLC in their urine. Whilst this increase would not be expected in all siblings of people with MS, the hypothesis was that those siblings with a high MS risk score would exhibit an increase in urinary FLC. It is already known that some siblings of people with MS exhibit CSF OCBs (92, 209), and the increase in urinary FLC was hypothesised to reflect this increased rate of CSF OCBs compared to controls.

Urinary FLC were therefore examined in all participants in this study who provided suitable urine samples (i.e. those without evidence of bacterial infection). Analysis was performed to determine both if the overall level of urinary FLC were increased in siblings of people with MS, and also if those siblings of people with MS who had a high MS risk score had significantly higher urinary FLC levels than those with lower MS risk scores.

6.2.1.1. Methods

6.2.1.1.1. Laboratory methods

The procedure for measuring urinary FLC is described in the methods, section 3.2.

Urinary kappa and lambda FLC were measured, allowing calculation of three ratios: urinary kappa FLC: protein, lambda FLC: protein and total FLC: protein.

6.2.1.1.2. Statistical analysis

Statistical analysis was performed using PASW 18 (SPSS). Variables were tested for normality using a Shapiro-Wilk test. As the urinary FLC:protein measurements were not normally distributed, an attempt was made to normalise them using a natural logarithmic transformation. However, this proved unsuccessful. The groups were therefore compared using non-parametric statistical methods. A Kruskal-Wallis Analysis of Variance was used to compare the three groups. The medians of the FLC:protein measurements were compared using the Independent Samples Median Test (k test). Post-hoc analysis using the Mann-Whitney U Test was performed to determine where the significant differences lay.

A possible correlation between the risk score and urinary FLC was assessed using the standard correlation coefficient, which does not make any assumption regarding the distribution of the data. Initially the risk score with genetic contribution from HLA-DRB1*1501 was used in this analysis, prior to a second analysis using the risk score with full genetic information. In order to compare the urinary FLC measurements between the 20 highest risk siblings and the 20 lowest risk siblings, the measurements for these samples were determined and then tested for normality. Again, the distribution of the urinary FLC:protein values was found to differ significantly from a Gaussian distribution and attempts to normalise this were unsuccessful. The values for those siblings in the highest risk score group were compared to the values for those siblings in the lowest risk score

group using the Mann-Whitney U Test, and the medians of the FLC:protein measurements compared using the Independent Samples Median Test (k test). Again, two analyses were performed, one using the risk score with genetic information from HLA-DRB1*1501 only, and one using the risk score with all genetic information.

In a final analysis, the values obtained for urinary FLC:protein for those siblings with an MS risk score above the median value were compared to those with a risk score less than the median. Again, the results for these samples were not normally distributed. They were therefore compared using the Mann-Whitney U Test and the medians compared using the Independent Samples Median Test (k test). Once again, two analyses were performed with the two different risk scores.

6.2.1.2. Results

6.2.1.2.1. Comparison between people with MS, siblings, and healthy controls

As stated above, the values for the FLC:protein ratios for people with MS, siblings and healthy controls could not be normalised. Non-parametric statistical analysis was therefore performed.

There was a significant difference between the groups overall for kappa FLC:protein (p=0.005), lambda FLC:protein (p=0.002) and total FLC:protein (p=0.004) (Kruskal-Wallis Analysis of Variance). There was also a significant difference between the medians for each group (kappa FLC: protein p=0.013, lambda FLC:protein p<0.0005, total FLC:protein p=0.004) (Independent Samples Median Test) (figures 6.10a-c; table 6.4).

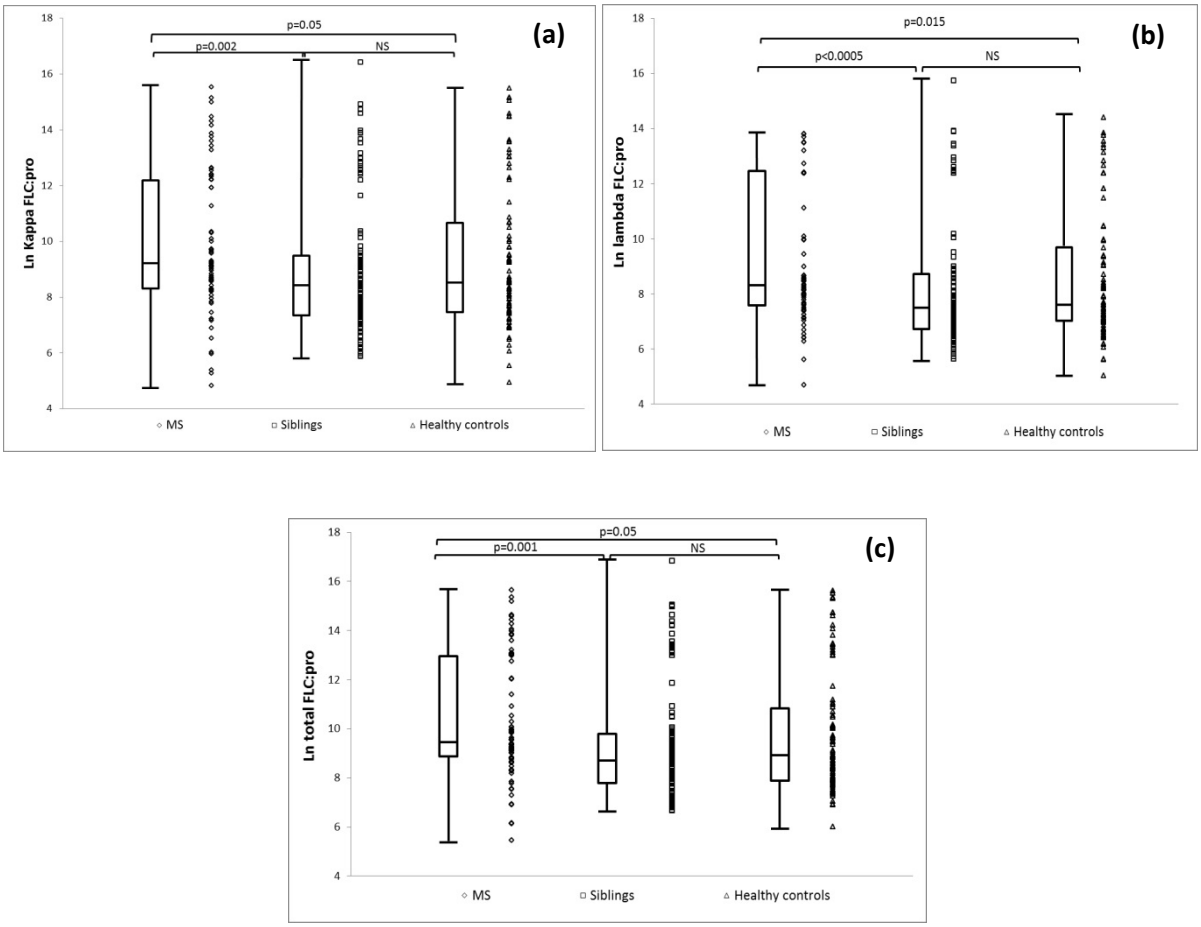


Figure 6.10: Combined box and whisker and scatter plot demonstrating the difference in urinary FLC:protein ratios . The box indicates the interquartile range, bisected by the median, and the whiskers the range. **(a)** Kappa FLC **(b)** Lambda FLC **(c)** Total FLC.

Table 6.4: Urinary free light chain:protein ratios

	MS (mean; SD)	Siblings (mean; SD)	HC (mean; SD)
Urine kappa FLC:protein (ug/l:mg/dL)	324,336 ($8.81x10^5$)[a]	249,625 ($1.29x10^6$)	317,211 ($9.16x10^5$)
Urine lambda FLC:protein (ug/l:mg/dL)	116,302 ($2.30x10^5$)[b]	125,243 ($6.41x10^5$)	102,624 ($2.74x10^5$)
Urine total FLC:protein (ug/l:mg/dL)	6,205,440 ($1.06x10^6$)[c]	376,936 ($1.93x10^6$)	419,836 ($1.16x10^6$)

a: Significant difference between people with MS and siblings (p=0.002) and healthy controls (p=0.05)
b: Significant difference between people with MS and siblings (p<0.0005) and healthy controls (p=0.015)
c: Significant difference between people with MS and siblings (p=0.001) and healthy controls (p=0.001)

On post-hoc analysis (Mann-Whitney U test) there was a significant difference between people with MS and their siblings with respect to kappa FLC:protein (p=0.002), lambda FLC:protein (p<0.0005) and total FLC:protein (p=0.001). There was a similar difference between people with MS and healthy controls (kappa FLC:protein p=0.05, lambda FLC:protein p=0.015, total FLC:protein p=0.05). There was no significant difference between the siblings of people with MS and healthy controls with respect to any of the FLC:protein measures (figures 6.10a-c; table 6.4).

6.2.1.2.2. Comparison between siblings with high MS risk score and low MS risk score

There was no correlation between FLC:protein measurements and overall MS risk score when the siblings of people with MS were studied in isolation (figure 6.11, data shown for risk score using HLA-DRB1*1501 only, similar results were obtained for risk score using full genetic information).

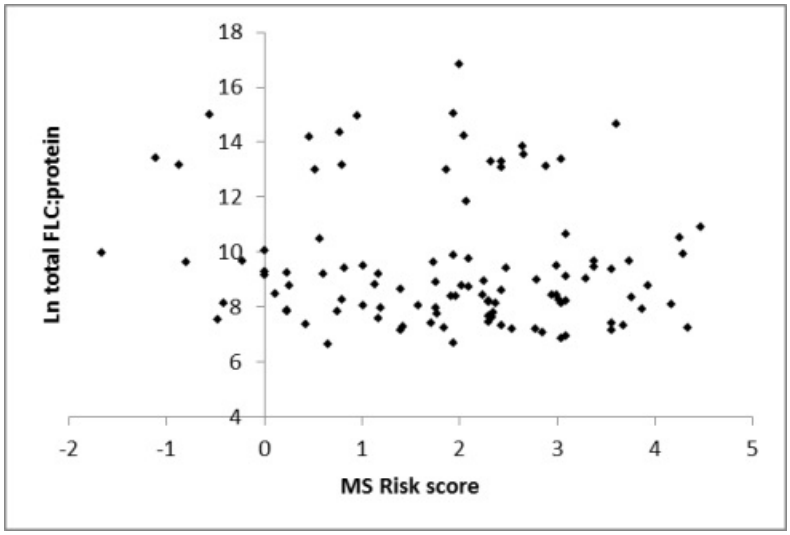


Figure 6.11: There is no relationship between MS risk score and total urinary FLC:protein ratio in siblings of people with MS. Similar appearances were seen for both kappa FLC:protein and lambda FLC:protein (data not shown). Data shown for risk score using HLA-DRB1*1501 only, similar results were obtained for risk score using full genetic information.

When those 20 siblings with the highest MS risk score were compared to those with the lowest MS risk score, there was no difference between the groups (Mann-Whitney U test) or the medians of the groups (Independent Samples Median Test) (figure 6.12a-c, data shown for risk score using HLA-DRB1*1501 only, similar results were obtained for risk score using full genetic information). When the sibling group was bisected around the median MS risk score and the urinary FLC:protein ratios compared between those with MS risk scores above the median and those with risk scores below the median MS risk score, there was no significant difference between the groups (figure 6.12d; data shown for total FLC:protein ratio only, similar findings were present for kappa FLC:protein and lambda FLC:protein, data not shown). Again, these results did not differ according to the risk score used.

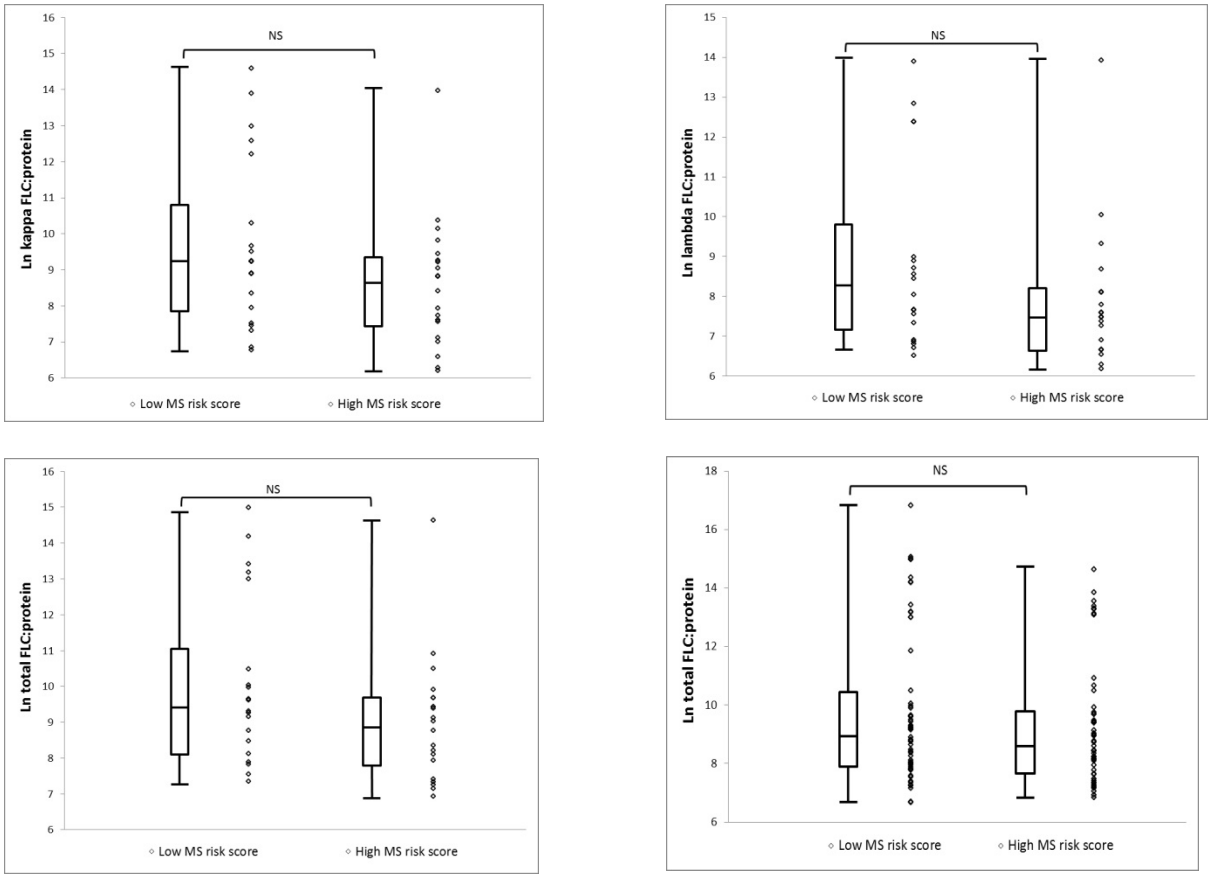


Figure 6.12: Combined box and whisker and scatter plot demonstrating the lack of difference in urinary FLC:protein ratios between those siblings with the lowest MS risk score and those with the highest MS risk score. The box indicates the interquartile range, bisected by the median, and the whiskers the range. Data shown for risk score using HLA-DRB1*1501 only, similar results were obtained for risk score using full genetic information. **(a)** Kappa FLC:protein ratios in the 20 siblings with the highest MS risk score and the 20 siblings with the lowest MS risk score **(b)** Lambda FLC:protein ratios in the 20 siblings with the highest MS risk score and the 20 siblings with the lowest MS risk score **(c)** Total FLC:protein ratios in the 20 siblings with the highest MS risk score and the 20 siblings with the lowest MS risk score **(d)** Total FLC:protein ratios in those siblings with an MS risk score lower than the median MS risk score compared to those siblings with an MS risk score greater than the median.

6.2.1.3. Conclusions

It can therefore be seen that the increased levels of urinary FLC:protein previously documented in MS (41, 43, 192) are present in this cohort of patients. The urinary FLC:protein levels are increased in people with MS compared to both healthy controls and their unaffected siblings.

However, levels of urinary FLC:protein do not significantly differ between siblings of people with MS and healthy controls. There is no indication that those siblings of people with MS with a higher MS risk score have increased levels of urinary FLC:protein when compared to those siblings with a lower MS risk score. This observation holds true regardless of whether those siblings with risk scores at the extremes are selected, or whether the entire sibling cohort is considered (i.e. bisected around the median). Similarly, when all siblings are considered, no relationship between MS risk score and urinary FLC:protein could be observed.

As discussed in chapter 3, the hypothesised relationship between urinary FLC:protein and CSF OCBs could not be confirmed. This was thought to be due to confounding by the possible presence of other systemic inflammatory conditions. The study of urinary FLC:protein in this sibling population has not shed any further light on the mechanism behind the increased levels seen in people with MS, nor has it been able to confirm an inflammatory endophenotype in those siblings of people with MS with a high MS risk score.

6.2.2. Urinary neopterin

6.2.2.1. Methods

6.2.2.1.1. Laboratory methods

The procedure for measuring urinary neopterin and creatinine is given in the methods, section 3.2. Urinary neopterin is expressed as a ratio to creatinine to correct for variable urine concentrations.

Due to financial constraints, only 20 MS samples and 20 HC samples were assayed. Samples were selected with MS samples having the 20 highest MS risk scores and HC samples having the lowest 20 risk scores. The entire sibling cohort was analysed. Details of the participants whose samples were assayed are given in the table in Appendix 7.

6.2.2.1.2. Statistical analysis

Statistical analysis was performed using PASW 18 (SPSS). Variables were tested for normality using a Shapiro-Wilk test. As the raw urinary neopterin:creatinine results were not normally distributed, they were normalised using a natural logarithmic transformation. Parametric statistical methods were used on the log transformed data.

An initial comparison of urine neopterin:creatinine between the MS samples, healthy control samples and sibling samples was carried out using a one-way analysis of variance (ANOVA). A post-hoc Bonferroni analysis was used to examine where the significant differences between groups lay.

The correlation between MS risk score and urine neopterin:creatinine in the siblings of people with MS was then assessed using the correlation coefficient. Two correlations were performed; one using the MS risk score including only the genetic contribution from HLA-DRB1*1501 and the other using the risk score generated using the full genetic dataset. The 20 siblings with the highest risk score for MS were then compared to the 20 siblings with the lowest risk score for MS. This was done according to both risk scores generated. Finally, the results for the siblings of people with MS were

divided according to the median MS risk score, and the neopterin:creatinine results compared. Again, this was performed according to both risk scores generated.

6.2.2.2. Results

6.2.2.2.1. Comparison between people with MS, siblings, and healthy controls

Although the raw values obtained for urinary neopterin:creatinine were not normally distributed, it was possible to convert them to a normal distribution using a natural logarithmic transformation. Parametric statistical tests were therefore used in the analysis.

One way analysis of variance (ANOVA) was used to compare urinary neopterin:creatinine between people with MS, their unaffected siblings and healthy controls. There was an overall significant difference between the three groups ($p<0.0005$, figure 6.13). When post-hoc analysis was performed to determine where the significant difference lay, there was a significant differences between urinary neopterin:creatinine between people with MS and their siblings ($p<0.0005$), and also between the siblings of people with MS and healthy controls ($p=0.010$) (figure 6.13). However, there was no significant difference between people with MS and healthy controls (figure 6.13).

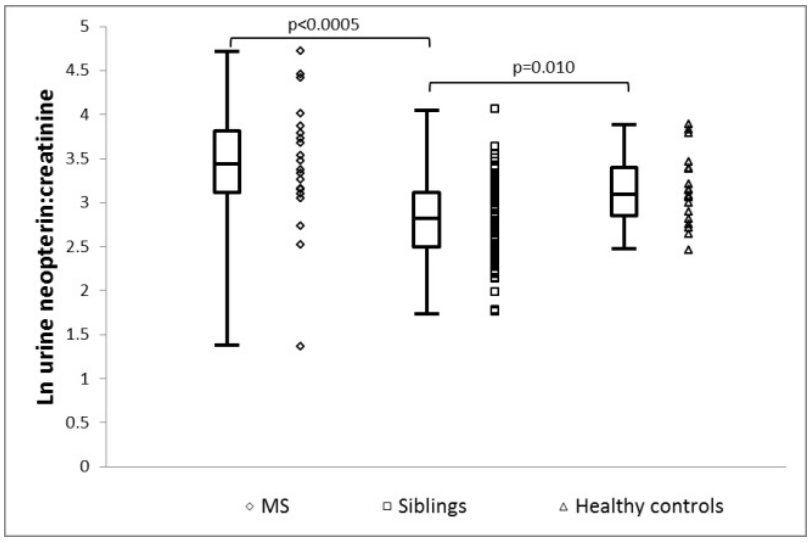


Figure 6.13: Combined box and whisker and scatter plot demonstrating the difference in urinary neopterin:creatinine ratios between people with MS, their siblings and healthy controls. The box indicates the interquartile range, bisected by the median, and the whiskers the range.

6.2.2.2.2. Comparison between siblings with high MS risk score and low MS risk score

When the siblings of people with MS were studied, there was no correlation between the MS risk score and urine neopterin:creatinine with either the risk score using HLA-DRB1*1501 only or the full genetic data (data not shown).

When the 20 siblings with the highest MS risk score were compared to the 20 siblings with the lowest MS risk score, there was no significant difference between the groups when the risk score using HLA-DRB1*1501 only was used (unpaired t-test) (figure 6.14a). However, when the risk score calculated using all genetic data was used to define the siblings with the highest and lowest MS risk scores, there did appear to be a significant difference in urine neopterin:creatinine between the two groups (p=0.017, unpaired t-test) (figure 6.14b). However, this difference was in the opposite direction to that hypothesised, with the siblings with the lowest MS risk score having a higher urine neopterin:creatinine than those with the highest MS risk score (figure 6.14b).

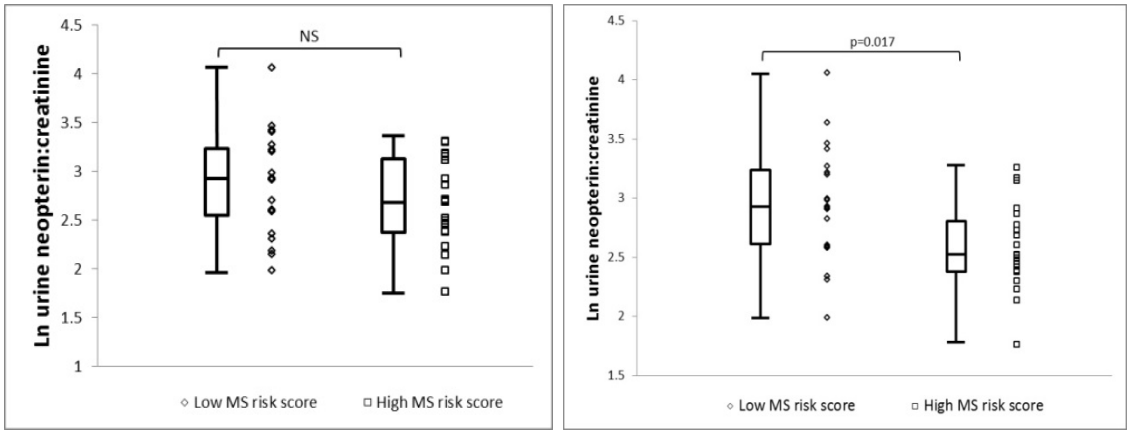


Figure 6.14: Combined box and whisker and scatter plot demonstrating **(a)** the lack of difference in urinary neopterin:creatinine ratios between those siblings with the lowest MS risk score and those with the highest MS risk score, when the risk score calculated using HLA-DRB1*1501 only was used. The box indicates the interquartile range, bisected by the median, and the whiskers the range. **(b)** The difference in urinary neopterin:creatinine ratios between those siblings with the lowest MS risk score and those with the highest MS risk score, when the risk score calculated using all genetic data was used. The box indicates the interquartile range, bisected by the median, and the whiskers the range.

When the entire sibling groups was bisected according to the median MS risk score, and the urinary neopterin:creatinine values for each half of the group compared, there was no significant difference between the two halves. This lack of difference was present regardless of the MS risk score used to order and bisect the sibling group (figures 6.15a and b).

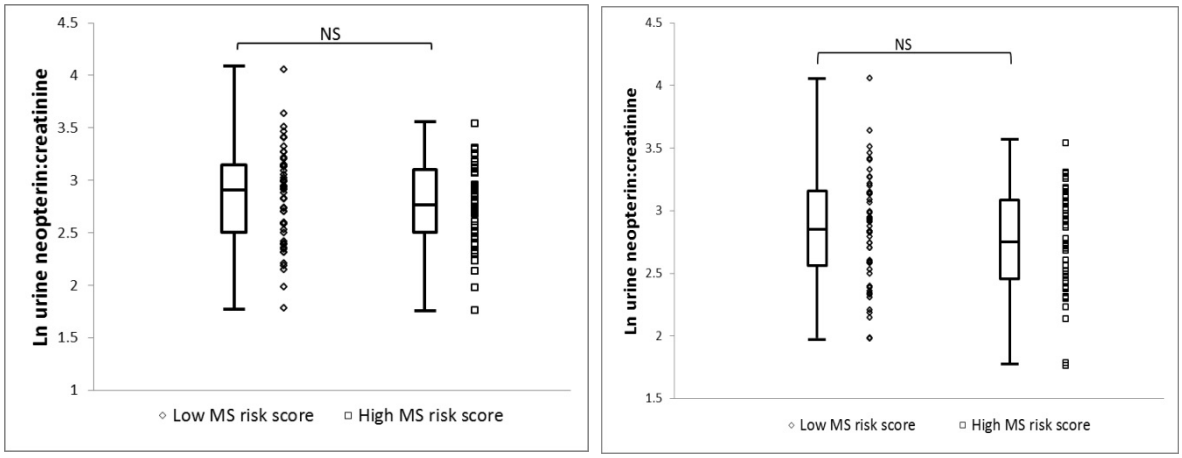


Figure 6.15: (a) Combined box and whisker and scatter plot demonstrating the lack of difference in urinary neopterin:creatinine ratios between those siblings with an MS risk score lower than the median MS risk score compared to those siblings with an MS risk score greater than the median, when the risk score calculated using HLA-DRB1*1501 only was used. The box indicates the interquartile range, bisected by the median, and the whiskers the range. **(b)** Combined box and whisker and scatter plot demonstrating the lack of difference in urinary neopterin:creatinine ratios between those siblings with an MS risk score lower than the median MS risk score compared to those siblings with an MS risk score greater than the median, when the risk score calculated using all genetic data was used. The box indicates the interquartile range, bisected by the median, and the whiskers the range.

6.2.2.3. Conclusions

It is interesting to note that whilst people with MS appear to have increased urinary neopterin:creatinine compared to their healthy siblings, this difference cannot be seen when comparing people with MS to the healthy controls in this study. However, this may be at least in part due to the fact that only 20 people with MS and 20 healthy controls were studied, rather than the full cohort. Whilst these samples were selected on the basis of the putative MS risk score (the 20 MS samples had high scores; the 20 healthy controls selected had low scores), this may not have been the best method by which to select samples, as the score has not yet been validated.

When studying the unaffected siblings of people with MS, the majority of the analyses did not reveal any difference in urinary neopterin:creatinine between those siblings with a high MS risk score and those with a low MS risk score. However, one analysis, that comparing the 20 siblings with the highest MS risk score to those with the lowest MS risk score determined using all genetic data, did show a significant result. This analysis appeared to show that urinary neopterin:creatinine levels were higher in those siblings with a low MS risk score compared to those with a high MS risk score. This finding, which is in the opposite direction to that hypothesised, is somewhat difficult to explain. The most likely explanation is that this is a type 1 error – i.e. an incorrect rejection of the null hypothesis. This type of error is possible in all experimental systems. Support for the theory that this result may represent a type 1 error comes from the lack of significant results when similar analyses are carried out on this dataset – namely comparing urinary neopterin:creatinine levels of the entire sibling group bisected by the median, or when using the risk score using HLA-DRB1*1501 only.

Of course, this result may not be the result of a type 1 error, and may be a truly significant result. Further work is required in order to validate this result, with a second group of siblings of people with MS. It may be that the lower neopterin:creatinine level seen in those siblings with high MS risk reflects the fact that although they have the genetic risk for MS, they do not carry the immunological phenotype for other reasons, and so have not gone on to develop the disease; whereas those with

lower genetic risk have the immunological phenotype in common with their affected siblings but they lack the genetic predisposition to disease.

6.2.3. Serum matrix metalloproteinase-9 (MMP-9)

6.2.3.1. Methods

6.2.3.1.1. Laboratory methods

When measuring serum MMP-9 levels, it is important to take into account the presence of tissue inhibitors of metalloproteinases (TIMP), the most relevant of which is TIMP-1. Thus, serum MMP-9 should be expressed as a ratio to TIMP-1, meaning that a measure of the active MMP-9 level that is used in all analysis.

The original publication describing increased an significantly increased MMP-9:TIMP-1 ratio in the serum of people with MS compared to healthy controls (228) compared 24 people with MS to 10 matched healthy controls. Power calculations derived from the values given by Waubant et al (228) for the MS and healthy control populations indicate that a sample size of 14 in each group (i.e. MS and healthy control groups) should be sufficient to demonstrate a difference between the groups. More recent studies have concentrated on the effect of treatment on MMP-9:TIMP-1 ratios in people with MS, and have not included a control group. As such, the sample size calculations were based on a relatively small study. Due to financial constraints, the decision was taken to use these power calculations and to perform this analysis on a subgroup of participants.

Twenty samples were selected for analysis from four groups: people with MS, siblings with a high MS risk score, siblings with a low MS risk score, and healthy controls. The risk score calculated using all genetic information was used for the selection of samples for this study. Details of the participants whose samples were assayed are given in the table in Appendix 7.

There is considerable debate as to whether MMP-9 and TIMP-1 should be measured in the serum or plasma. Although detectable in both, sampling techniques mean that levels can differ considerably (231-233). For the reasons discussed in section 4.2.12.2, in this study levels were measured in the serum. The methods for measuring both MMP-9 and TIMP-1 are given in section 4.2.12.2.

6.2.3.1.2. Statistical analysis

Statistical analysis was performed using PASW 18 (SPSS). Variables were tested for normality using a Shapiro-Wilk test. As the raw MMP-9:TIMP-1 ratios were not normally distributed, they were normalised using a natural logarithmic transformation. Parametric statistical methods were used on the log transformed data.

An initial comparison of MMP-9:TIMP-1 ratios between the MS samples, healthy control samples and sibling samples was carried out using a one-way analysis of variance (ANOVA). A post-hoc Bonferroni analysis was used to examine where the significant differences between groups lay.

As the number of samples analysed was relatively small, the decision was made not to examine for a correlation between MMP-9:TIMP-1 ratio and MS risk score.

6.2.3.2. Results

There was no significant difference between any of the groups with respect to MMP-9:TIMP-1 ratio, either when the sibling samples were considered as a whole, or when they were split according to the MS risk score (figure 6.16).

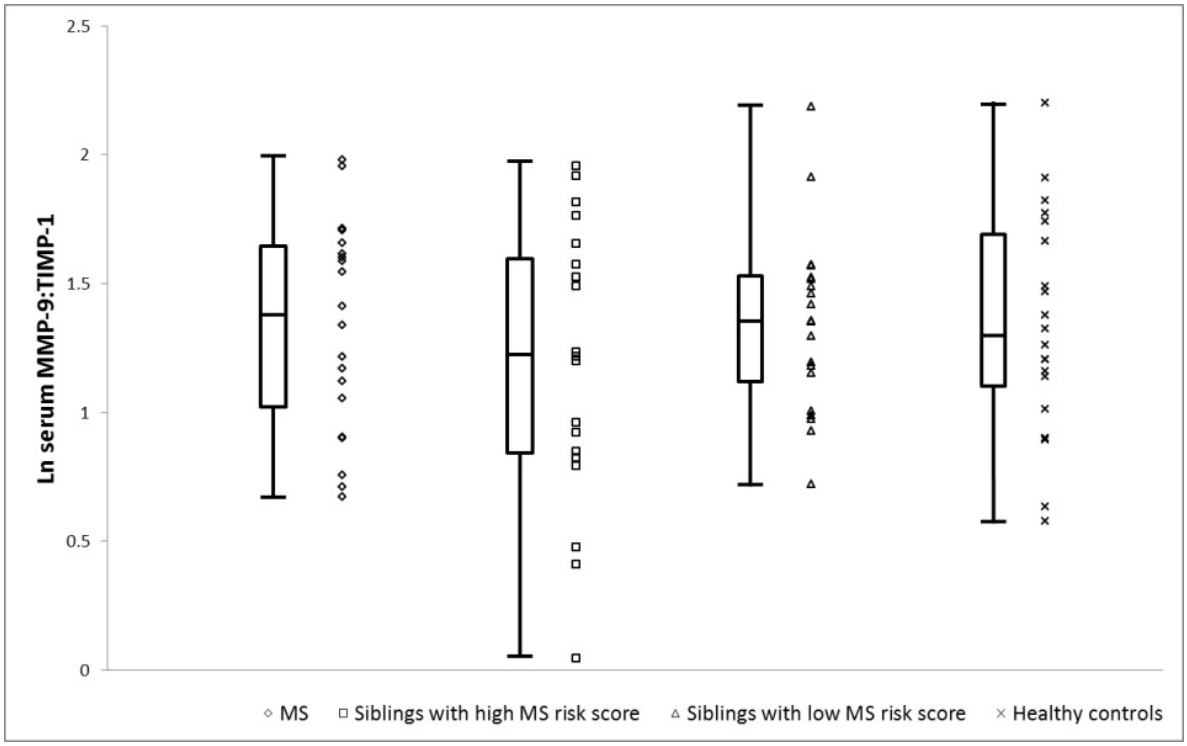


Figure 6.16: Combined box and whisker and scatter plot demonstrating the lack of difference in serum MMP-9:TIMP1 ratios between people with MS, their siblings with high and low risk scores, and healthy controls. The box indicates the interquartile range, bisected by the median, and the whiskers the range.

6.2.3.3. Conclusions

The previous findings of increased serum MMP-9:TIMP-1 ratios in MS (228, 229) are therefore not confirmed in this study. In addition, there was no appreciable difference between MMP-9:TIMP-1 ratios between either of the sibling cohorts and people with MS or healthy controls. Additionally, there was no significant difference between those siblings with high MS risk score and low MS risk score.

These findings may be due to a genuine lack of difference in MMP-9:TIMP-1 ratio between people with MS and healthy controls – previous studies have used either plasma or serum, however there does not appear a correlation between the two (338). Additionally, not all studies have used the MMP-9:TIMP-1 ratio as a marker of "active MMP-9", with others using alternative techniques (339). The existing literature is therefore inconsistent in terms of methodology, which leaves the results generated difficult to interpret. .

A further possibility is that the use of serum samples adversely affected the results. As previously stated (section 4.2.12.2) (338), there is no consensus as to whether serum or plasma should be used for MMP-9 measurement. In the samples collected as part of this study, it was not possible to measure plasma MMP-9:TIMP-1 ratios as the plasma samples were collected in lithium heparin, which affects the concentration of TIMP-2 (234), and therefore is quite likely to affect TIMP-1.

Further work is therefore needed both to determine the optimal biological fluid for the measurement of MMP-9 and TIMP-1 levels, and to develop an operating procedure for sample collection, processing and storage that provides reproducible and accurate readings. Once this has been developed, then the true differences (or lack of) between MMP-9:TIMP-1 ratios in MS can be determined, and its use as a biomarker of disease activity formally evaluated.

6.3. Flow cytometry

6.3.1. Methods

6.3.1.1. Laboratory methods

Flow cytometry analysis was performed on a subgroup of samples. Cells were thawed from 8 people with MS who had a high MS risk score and were not on disease modifying therapy, 8 unaffected siblings of people with MS with high MS risk scores, 8 unaffected siblings with low MS risk scores and 8 healthy controls with low MS risk scores. Details of the participants whose samples were assayed are given in the table in Appendix 7.

As described in section 4.2.13 the proportion of CD3+/CD4+ cells demonstrating positive staining for intracellular IL-17 and FoxP3 was determined. The proportion of cells demonstrating positive staining was determined both prior to stimulation and following stimulation with PMA and ionomycin, and the increase in the proportion of cells demonstrating positive staining used in all analysis. Both the raw change in IL-17 and FoxP3 expressing cells and the percentage change were compared between groups. All flow cytometry analysis was performed using FlowJo software.

6.3.1.2. Statistical analysis

All statistical analysis was performed using SPSS. Both the absolute change and percentage change between unstimulated and stimulated cells were found to be normally distributed. Parametric statistical methods were therefore used. The four groups were compared using a one-way ANOVA, and post-hoc pairwise analysis was performed with a Bonferonni correction.

The raw results for IL-17 measured in the cell culture supernatant were not normally distributed. However, they were normalised using a natural logarithmic transformation; therefore parametric statistical tests were used on the log transformed data. The medians of the four groups were examined for a trend across the groups using linear regression modelling.

6.3.2. Results

The mean change in the proportion of cells demonstrating positive staining is given in table 6.5. There was no significant difference in either absolute or percentage change in the proportion of cells expressing IL-17 (figure 6.17). There was a significant difference overall between the three groups in the percentage change in the proportion of cells demonstrating FoxP3 expression between the four groups; however none of the differences were significant on pairwise testing (figure 6.18). There was no difference between the groups in the proportion of cells expressing both IL-17 and FoxP3.

There was no overall significant difference between the groups in the IL-17 concentration measured in the cell culture supernatant (figure 6.19). No significant difference was detected between the groups on pairwise testing. However, when the medians of the groups were examined, there was a significant trend towards reducing IL-17 in cell culture supernatant with reducing MS risk scores (i.e. greater levels of IL-17 in cell culture supernatant from people with MS) (figure 6.19 and table 6.5).

Table 6.5: Absolute and percentage change in the proportion of cells staining positive for IL-17, FoxP3, or both.

	MS	Siblings (high MS risk score)	Siblings (low MS risk score	Healthy controls
Mean change in proportion of cells expressing IL-17 (mean; SD)	1.68 (1.19)	1.78 (1.42)	1.17 (0.77)	2.07 (0.66)
Mean % change in proportion of cells expressing IL-17 (mean; SD)	101.29 (85.29)	341.12 (309.99)	221.01 (148.51)	291.18 (263.74)
Mean change in proportion of cells expressing FoxP3 (mean; SD)	0.28 (0.32)	0.53 (0.34)	0.42 (0.38)	0.14 (0.24)
Mean % change in proportion of cells expressing FoxP3 (mean; SD)[a]	17.84 (22.03)	40.83 (24.93)	43.22 (36.74)	11.16 (18.39)
Mean change in proportion of cells expressing both IL-17 and FoxP3 (mean; SD)	0.19 (0.12)	0.15 (0.08)	0.11 (0.06)	0.19 (0.08)
Mean % change in proportion of cells expressing both IL-17 and FoxP3 (mean; SD)	196.84 (164.25)	298.40 (166.47)	314.74 (242.92)	344.12 (230.45)
Mean IL-17 in cell culture supernatant (pg/ml) (mean; SD)	37.87 (8.14)	40.65 (25.51)	30.66 (7.47)	27.68 (4.82)

a: Overall significant difference between four groups, not maintained on pairwise testing with Bonferroni correction.

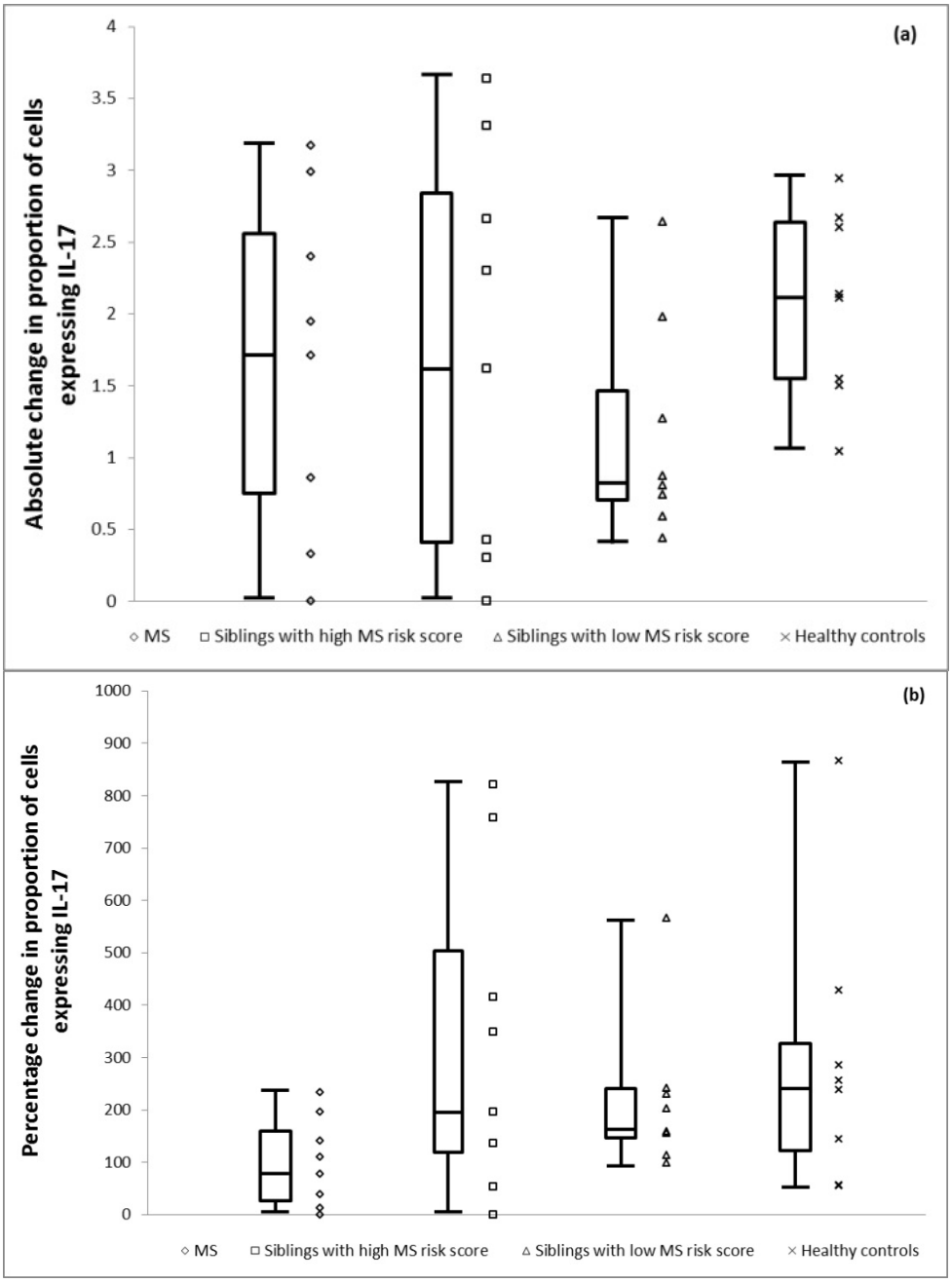


Figure 6.17: (a) Combined box and whisker and scatter plot demonstrating the lack of difference in absolute change in intracellular IL-17 expression between people with MS, their siblings with high and low risk scores, and healthy controls. The box indicates the interquartile range, bisected by the median, and the whiskers the range. **(b)** Combined box and whisker and scatter plot demonstrating the lack of difference in percentage from baseline change in intracellular IL-17 expression between people with MS, their siblings with high and low risk scores, and healthy controls. The box indicates the interquartile range, bisected by the median, and the whiskers the range.

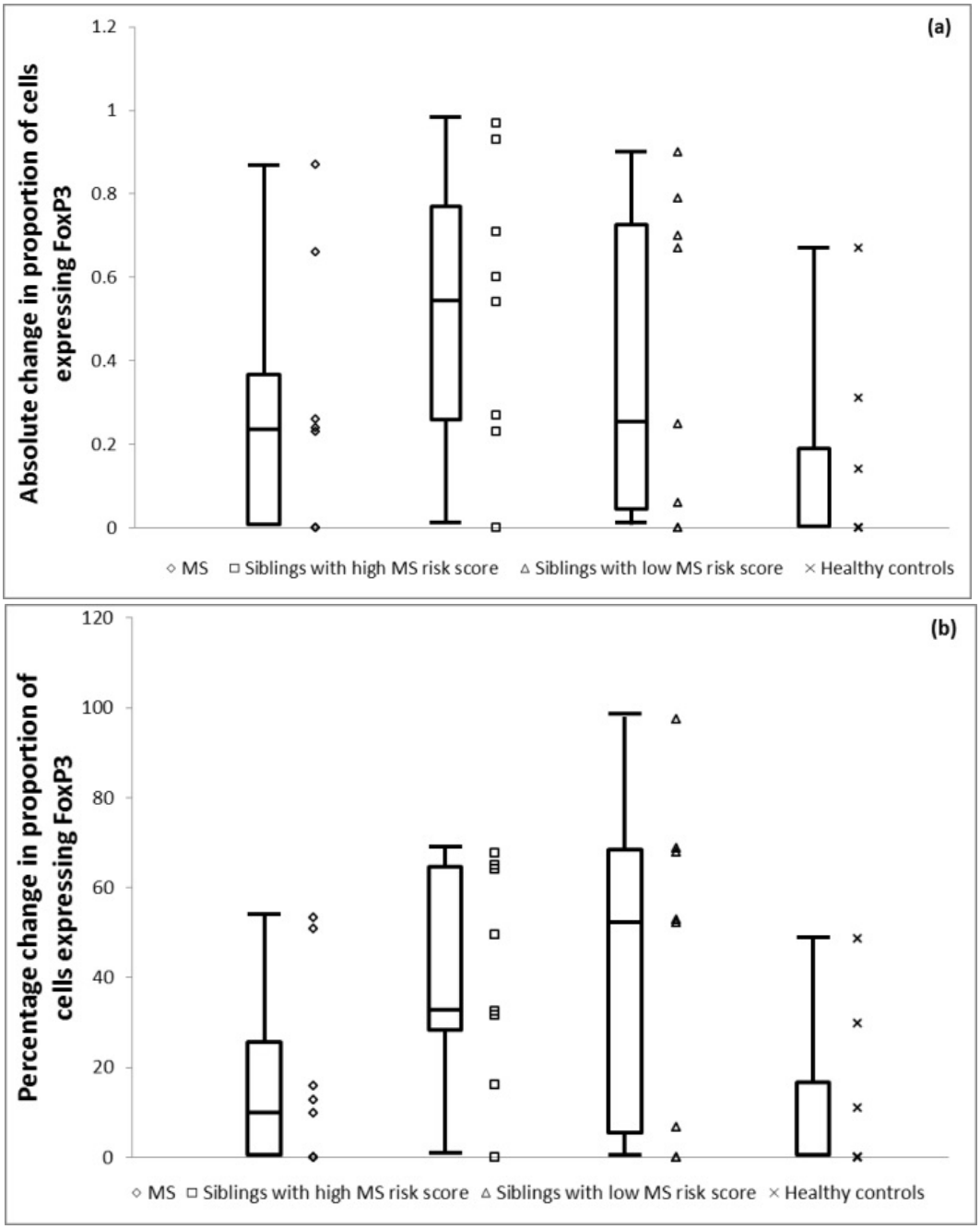


Figure 6.18: (a) Combined box and whisker and scatter plot demonstrating the lack of difference in absolute change in intracellular FoxP3 expression between people with MS, their siblings with high and low risk scores, and healthy controls. The box indicates the interquartile range, bisected by the median, and the whiskers the range. **(b)** Combined box and whisker and scatter plot demonstrating the difference in percentage from baseline change in intracellular FoxP3 expression between people with MS, their siblings with high and low risk scores, and healthy controls. No significance was demonstrated on pairwise testing with Bonferonni correction. The box indicates the interquartile range, bisected by the median, and the whiskers the range.

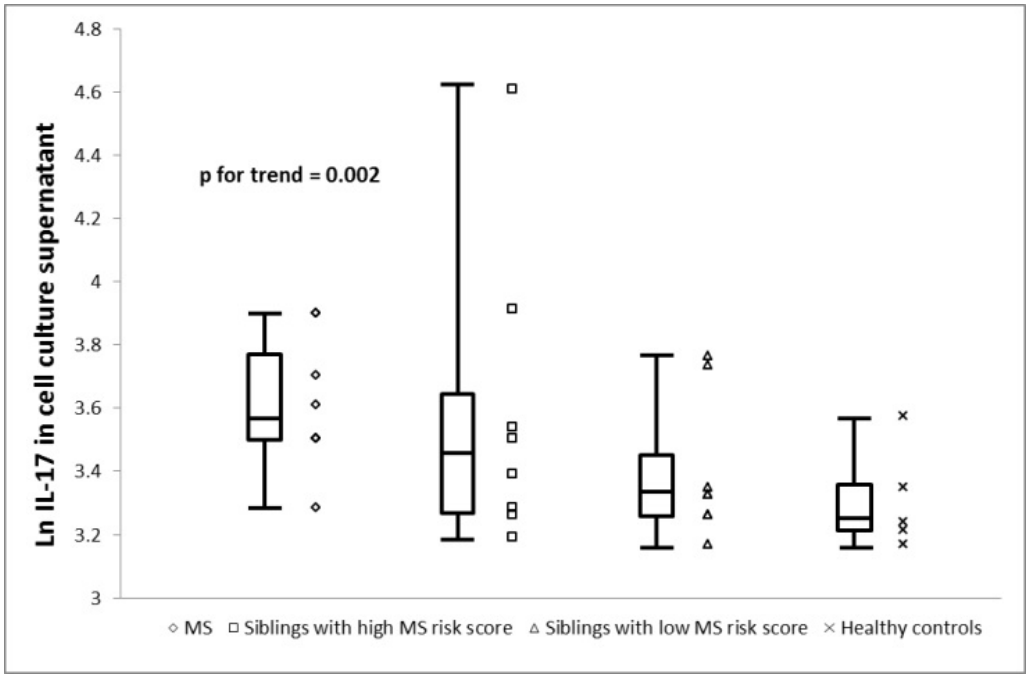


Figure 6.19: Combined box and whisker and scatter plot demonstrating significant trend towards a reduction in median IL-17 levels in the cell culture supernatant between people with MS, their siblings with high and low risk scores, and healthy controls. The absolute differences between the groups were non-significant. The box indicates the interquartile range, bisected by the median, and the whiskers the range.

6.3.3. Conclusions

It can therefore be seen that whilst there does not appear to be a significant difference or trend between the MS risk score groups in the number of cells expressing intracellular IL-17 or FoxP3, there does appear to be a trend in the IL-17 levels in the cell culture supernatant. The number of samples used in this analysis is relatively small; however, any potentially significant trend should have been apparent at this level. The variation in the increase in both intracellular IL-17 and FoxP3 within each of the groups is relatively large, and this may explain in part the lack of significant results noted.

However, the cell culture IL-17 results are more convincing. Whilst the differences between the groups are sufficiently small as to render the absolute differences non-significant, the trend across the medians is clear, and requires validation. It does, however, provide some evidence potentially supporting the validity of the putative MS risk score.

6.4. MRI

6.4.1. Methods

The MRI imaging substudy was performed as a pilot study within the overall study. Extrapolating from existing data on the proportion of siblings who would be expected to demonstrate MRI abnormalities in keeping with demyelination, approximately 10% of the siblings enrolled in this study would be expected to demonstrate these changes (93, 205). This equates to approximately 10 unaffected siblings with MRI evidence of demyelination. If the MS risk score is indeed a marker of the MS endophenotype (which includes those with radiological evidence of demyelination), then those siblings with high MS risk score would be expected to demonstrate MRI changes, whilst those with a low MS risk score would not. Assuming those siblings demonstrating MRI changes would be clustered in the qunitile with the highest MS risk score, this would give a potential frequency of MRI changes in this quintile of up to 50%. If this assumption is correct, given a background rate of 0.06% of asymptomatic MRI changes in keeping with demyelination (206), it was decided to image 12 siblings with high MS risk scores and 12 with low MS risk scores.

A total of 40 siblings (20 with the highest MS risk scores and 20 with the lowest MS risk scores) were invited to attend for MR imaging. The intention was not to image all of these siblings, but to allow for dropout rates. One sibling, who had one of the highest MS risk scores, had developed clinically definite MS between the initial visit and being invited to attend for MRI; the decision was taken not to perform additional imaging on this participant.

A total of 22 siblings (12 with high MS risk score and 10 with low MS risk score) had MR imaging performed at the Institute of Neurology. Details of scan acquisition and image interpretation are given in section 4.2.14. One additional sibling with low MS risk score was able to provide a recent MRI scan that had been performed elsewhere. This participant was unable to have a repeat MRI

scan due to recent surgery, therefore the scan provided by this sibling was used for analysis. One further sibling with low MS risk score attended for MRI examination but was unable to proceed with the scan on safety grounds.

6.4.2. Results

Details of the participants who underwent MRI imaging is given in table 6.6. There were significantly more males in the low MS risk score group; however this was not unexpected due to the contribution of gender to the MS risk score. There was no significant difference between the groups in terms of age.

Table 6.6: Details of siblings who underwent MR imaging

	Siblings with high MS risk score	Siblings with low MS risk score
Number	12	11
Age (mean; SD; range)	48.8 (11.1; 26-67)	53.2 (11.0; 31-69)
Gender (M:F; %F)	2:10 (83.3% F)	6:4 (40.0% F)
MS risk score (genetic contribution from HLA-DRB1*1501) (mean; SD)	3.74 (0.50)	-0.26 (0.75)
MS risk score (full genetic information) (mean; SD)	10.80 (0.61)	6.42 (0.79)
Number of participants with any hyperintensities on T2 weighted MRI (n; %)	10/12 (83.3%)	7/10 (70.0%)
Number of participants with supratentorial T2 hyperintensities on MRI (n; %)	10/12 (83.3%)	7/10 (70.0%)
Number of participants with infratentorial T2 hyperintensities on MRI (n; %)	0/12 (0%)	2/10 (20.0%)
Number of participants with juxtacortical T2 hyperintensities on MRI (n; %)	2/12 (16.7%)	1/10 (10.0%)
Number of participants with periventricular T2 hyperintensities on MRI (n; %)	3/12 (25.0%)	2/10 (20.0%)
Mean number of hyperintensities on T2 weighted MRI (mean; SD; range)	8.7 (5.6; 0-17)	10.5 (15.2; 0-40)
Number of participants judged to have "possible demyelination" on MRI (n; %)	1/12 (8.3%)	1/10 (10.0%)
Number of participants judged to have "likely demyelination" on MRI (n; %)	0/12 (0%)	0/12 (0%)
Number of participants who developed clinically definite MS (CDMS)	1	0

6.4.3. Conclusions

There was no significant difference between the groups in terms of number or location of lesions. One participant in the group with high MS risk score had developed clinically definite MS by the time of imaging, and this participant did not have exploratory imaging, so is not included in the rest of the data in table 4.42. As can be seen from the results, one participant in each group had changes that could be described as "possible demyelination" according to accepted criteria.

Although the MRI results were not statistically significant, it is entirely possible that this is due to statistical underpowering of this part of the study. A total of 3 siblings selected for imaging studies (i.e. of the 40 initially selected) demonstrated evidence of demyelination, this is close to the 10% predicted from other studies. There may be more of the 40 siblings who were contacted but who did not undergo MRI that show asymptomatic abnormalities.

One sibling developed clinically definite MS after their initial visit; their MS risk score was one of the 10 highest scores in the sibling group. The sibling in the high MS risk score group who demonstrated "possible demyelination" on MRI also had one of the 10 highest MS risk scores. The sibling in the low MS risk score group who had MRI changes was actually the siblings with the highest score in the low MS risk group.

Hyperintense T2 lesions are the most frequently used and well validated measure of demyelination. There is some evidence of other early changes on MRI spectroscopy in patients with RIS (340), with patients demonstrating reduced NAA/Cr levels compared to healthy controls. There is also evidence of grey matter changes at the time of clinically isolated syndrome (341). These grey matter changes do not fully correlate with hyperintense T2 lesions (342), and so should be examined for in addition to the analysis performed above.

Chapter 7: Overall Conclusions

The highlight of this study is the development of a MS risk score that demonstrates an area under the ROC curve (AUC) of 0.82. Indeed, the figure of 0.82 approaches what is generally thought of as a clinically useful figure of 0.85. A multivariate risk score for MS carries the potential to allow improved information for both patients with clinically isolated syndromes and also for those at high risk of developing MS. At present, the overwhelming majority of US neurologists are of the opinion that they have evaluated a patient who has been misdiagnosed with MS in the past year (36). Given the recent and ongoing changes in the treatment landscape, with more efficacious therapies being developed at the cost of significant potential risk to patients, making an accurate diagnosis of MS is more important now than ever before. If an MS risk score can be integrated into the diagnostic pathway alongside the existing clinical diagnosis then both accuracy of diagnosis and potential early access to treatments may improve.

At present, as a general rule, patients have to wait to have two relapses before either gaining a clinical diagnosis or access to disease modifying therapy in the UK. There is no single diagnostic test for MS, which has potentially limited the access to disease modifying treatments for those with early stage disease. It is increasingly being recognised that “time is brain” even within the field of MS, and early treatment is likely to have long-term effects on disability outcomes. An MS risk score, which could provide additional risk stratification to MRI and CSF findings for those presenting with CIS, may aid the identification of those who need closer monitoring and follow-up. It may be that this risk score is able to provide information over and above that provided by MRI and CSF findings regarding the risk of converting from CIS to CDMS, allowing targeted therapy at clinical disease onset for those at high risk of future relapses.

This risk score can also contribute to our understanding of the causal cascade that eventually results in MS onset in adulthood. Including all genetic variants identified by the 2011 GWAS (103) considerably improved the AUC for the MS risk score. This highlights the importance of the genetic

underpinning of this complex disease. Through the development of this risk score, and the comparison to other similar scores which include fewer genetic variants, it can be seen that the AUC steadily improves as increasing numbers of SNPs that have been associated with MS are included. However, given the relatively modest improvement in the AUC with the inclusion of a large number of additional SNPs it seems unlikely that expanding the genetic contribution to the score will eventually result in a "perfect" score. Instead, it is likely that further refinement of the influence of the environment, which is influenced by the genetic makeup of an individual will result in meaningful improvement in such a score. From the results obtained in this study, it seems unlikely that MS is either a purely genetic nor a purely environmental disease; rather the two coexist and interact in a multitude of ways, many of which we currently lack the means to study.

The majority of the biomarkers studied did not demonstrate the hypothesised gradient along the MS risk score. However, there was a trend to a reduction in IL-17 levels in the cell culture supernatant across reducing levels of risk score. It must be noted that this sub-study was performed in a low number of participants, and these findings require replication. Despite the caution regarding the preliminary nature of these findings, this novel discovery potentially sheds light on early peripheral changes that may be associated with MS. Th17 cells are a relatively recent avenue of interest in the field of MS, with increased levels of IL-17 seen in MS, particularly in the context of relapses (161, 162, 163). Indeed, on a microscopic level, transcripts encoding IL-17 were found to be elevated in MS plaques when compared to control brains (370). IL-17 acts as a potent pro-inflammatory cytokine, upregulating the production of other cytokines including IL-1 and IL-6 (169), thus placing IL-17 in a potentially important place in the cascade of inflammation that appears to result in clinical disease. However, it is unlikely that IL-17 is the only part of the jigsaw – mice treated with anti-IL-17 antibodies demonstrate a delayed disease onset and/or a reduction in the severity of EAE (371-2), the animal model of MS. However, they still go on to develop the disease, highlighting the multifactorial nature of EAE and MS.

Given the mainly negative findings of the rest of the peripheral biomarker work, it would appear that widespread subclinical inflammation is unlikely to be part of the endophenotype. This does not completely exclude such changes in individuals with early (i.e. asymptomatic) demyelination; this study was not sufficiently powered to study this, and a larger study focussing purely on individuals with MRI evidence of active demyelination would be required. These changes may be relatively downstream of the early immunological endophenotype possibly typified by increased IL-17 secretion in response to Th17 cell stimulation.

The fact that there were no statistically significant differences between the two cohorts who underwent MRI does not mean that the endophenotype concept is not applicable to MS. A relatively small number of participants were imaged, and it may be that imaging the entire cohort is needed to fully assess the distribution of risk. Hyperintense lesions seen on T2 MRI are the hallmark of demyelination, and underpin our understanding of MS. These lesions represent focal areas of demyelination, and follow a characteristic pattern in MS. In relapsing remitting MS, new T2 lesions are the best indication of subclinical active disease that we have, and such changes are widely used as the outcome measures in clinical trials of disease modifying therapy. In patients with CIS, those with MRI changes in keeping with MS at the time of presentation demonstrated a hazard ratio of either 6.1 (patients with 1 or 2 Barkhof criteria) or 17.0 (patients with 3 or 4 Barkhof criteria) for conversion to CDMS compared to those with no lesions within a median of 7 years (373).

Data on patients with asymptomatic changes in keeping with demyelination (RIS) is limited at present. As access to MRI for a range of indications outside MS improves, the number of people with RIS can only increase. At present, it is difficult to know what to tell such patients – if they have never had any clinical symptoms that would suggest a clinical diagnosis of MS then clearly this diagnosis cannot be made. The risk of progression to clinical symptoms is not accurately known – to date clinical follow up of these patients has been of limited numbers and relatively short duration. Using the available data, which is based on studies of subjects with a relatively high lesion load at baseline,

approximately a third of patients with RIS appear to go on to develop clinical symptoms (i.e. CIS) within about 2 years; a proportion of these then develop a second clinically apparent demyelinating event and are diagnosed with clinically definite MS (367). The group with asymptomatic MRI changes in keeping with demyelination provide an ideal cohort for further investigation of both the endophenotype concept and the MS risk calculator developed within this thesis – this will be discussed further in chapter 9.

More novel quantitative work may be able to provide more insight into very early changes, such as changes seen on MRI spectroscopy, or measures of grey matter changes (340, 341). Analysis of such changes may be able to provide not only evidence of early disease, but also an insight into the early development of MS. However, this work remains highly speculative and is far from diagnostic.

Another limitation of the MRI step was the number of participants who underwent imaging. MRI imaging of asymptomatic siblings is not without ethical and practical concerns. Whilst no asymptomatic clinically significant abnormalities (outside of demyelination) were discovered, the risk of uncovering such abnormalities is estimated to be around 1.7-4.3%, depending on the resolution and sequences performed (206). The cost and practicalities of imaging large numbers of participants are also a limiting step, and hence the decision was taken to only perform MRI on a subgroup of siblings. Clearly the power of this part of the study could be improved by increasing the number of participants undergoing imaging studies.

One question that it is important to answer is whether OCB negative MS is truly MS. In primary progressive MS, the presence of OCBs is enshrined within the diagnostic criteria; the same is not true of relapsing remitting MS. OCBs have had a somewhat chequered history, with inter-assay variability and poor reproducibility significantly hampering research. However, with the advent of IEF and immunostaining, these practical considerations have effectively been overcome. The relatively high proportion of neurologists who are of the opinion that they have evaluated a misdiagnosed patient

within the past year indicates the need for robust diagnostic criteria, and a re-examination of the clinical phenotype of the "OCB negative MS" is required. A significant proportion of this group almost certainly do not have MS, and whether the remainder convert to being OCB positive is not known. Given the success of B-cell modulating therapies in MS (173-175), and the increasing evidence that meningeal B-cells are present in the brains of people with MS (176, 177), we cannot continue to ignore this group. If they have an alternative underlying cause of demyelination, it may be that current treatments for MS are not the correct treatment for these patients (as is the case in neuromyelitis optica, NMO). If, as I suspect, they have a different demyelinating disease, which is associated with a different causal cascade, this tells us something very important about MS and the factors relevant to disease development.

Are there other factors that could be incorporated into disease prediction using an MS risk score? A potentially important contributor to MS risk that is not detected by GWAS is relatively rare alleles associated with large effect sizes. These low frequency variants are not present on current SNP genotyping arrays. It is becoming increasingly clear that rare variants are more common than initially thought (105), suggesting that individual disease risk may be influenced by rare or indeed private (confined to one individual/family) mutations. Examples of these in MS have recently been described and it is extremely likely that more will follow (104, 106). Discovering these variants will require genome sequencing in large patient numbers; whilst this is rapidly becoming affordable, it is unclear how useful this would in terms of disease prediction as this depends on how frequent these rare alleles actually are.

GWAS are underpowered to detect gene-gene interactions. There is evidence of some interaction at the HLA locus in the context of MS (343) and there may be other interactions yet to be discovered, which will almost certainly add to AUC values once incorporated into any disease prediction model.

Gene-environment interactions, which almost certainly play a role in MS susceptibility, are missed by GWAS. The effect of gene-environment interactions on the strength of genetic contribution to disease is difficult to estimate outside studies examining specific relationships between defined genetic markers and environmental exposures. Given the relatively small effect of both genetic variants and environmental contribution to disease risk in the context of MS, studies to examine a potential gene-environment interaction would have to be a multinational collaboration over many tens of years; there are obvious problems with the feasibility of any such study. An illustrative model implied a marked potential effect of such interactions on the OR calculated by GWAS, with an increase in the OR of up to 16.8 from a GWAS estimated OR of 1.3 (344). This model does not take account of the magnitude of the effect of any environmental exposure, instead assuming a binary relationship, limiting the direct applicability of the results. Whilst it seems highly likely that such interactions play a significant role in modulating disease risk, studying this through a purely epidemiological approach has severe limitations, and alternative approaches such as epigenetic correlations will need to be used.

Environmental influences on genetic risk, many of which are likely to be mediated through epigenetic mechanisms, are complex to include into such a prediction algorithm. The parent-of-origin effect in MS (98), which has been highlighted as a demonstration of potential unknown influences on genetic risk, is well described, and discussed in section 4.1.2.2. The precise mechanisms underlying this apparent differential transmission of risk remain speculative, but are likely to involve epigenetic changes, which are likely to at least partly explain the discordance in MS seen in genetically identical monozygotic twins. If GWAS were fully able to predict MS susceptibility, MZ twin concordance rates would approach 100%; however, it is around 25% (3). Outside of MS, epigenetic differences between MZ twins have been demonstrated (109). To date, only one study has examined methylation status in MZ twins discordant for MS (110), and this did not uncover any differences. However, this study had an extremely small sample size (n=3), and this, together with

the high stringency measures, mean that there is more work to be done in this arena. Epigenetic differences will undoubtedly add to risk prediction once they are uncovered.

There therefore remains much work to be done, both in terms of the validation of this MS risk score in a larger cohort, but also with respect to the discovery, validation and incorporation of additional MS risk factors and risk factor interactions into the putative MS risk score. However, there is the potential for this risk score to be used in order to test hypotheses regarding MS development and it has the potential to enable directed preventative studies.

Chapter 8: Discussion

If it is indeed possible to define MS risk according to the MS risk score described above, then an important potential use for this would be in disease prediction and the enrichment of pre-symptomatic prevention studies with people at high risk of MS. Such prevention studies could take the form of early, and possibly on-going, intervention(s) with longitudinal follow-up to assess rates of disease development. It is clear from existing evidence, together with the findings from this study, that the siblings of people with MS represent a population loaded with genetic risk for MS. The environmental factors measured in this study, which have previously been linked to MS and extensively validated, would seem to be reasonable initial targets for intervention.

As much of the non-genetic MS risk present in an individual appears to be essentially non-modifiable (such as the risk associated with place and month of birth, maternal factors and gender), there are a relatively few factors that can be modified during an individuals' lifetime – namely vitamin D status, factors related to Epstein-Barr virus infection, and smoking.

We live in a world where vitamin D deficiency has become the norm. Changes in lifestyle and behaviour in the developed world have led to what has been described as an "epidemic of vitamin D deficiency" (345). Due to safety concerns our children no longer play outside, instead socialising online. Societal pressures and targeted marketing have led to an explosion in the use of sunscreens: cosmetic products now proudly proclaim their sun protection factor (SPF) as a positive attribute. Our working patterns mean that many no longer see the sun during the working week, instead remaining indoors, glued to computer screens. Despite this, the recommended daily allowance (RDA) for vitamin D has not changed its inception.

The first attempt to develop recommended values for dietary vitamin D intakes was in 1941. The value of 400 IU/day (10ug) was chosen; derived by measuring the amount of vitamin D in a teaspoon of cod-liver oil (346, 347). Over the intervening 70 years, the RDA has not changed significantly

(table 8.1). The argument for maintaining the status quo has been based in the arena of bone health: the Institute of Medicine (IOM) in the USA state that a serum level of 25(OH)vD of 12.5 ng/ml (30nmol/l) is sufficient to maintain bone health (348, 349). The acceptable intake (AI; equivalent to the UK RDA) figures for the USA are therefore based on maintaining serum 25(OH)vD levels of 27.5nmol/l in the absence of sunlight (349). This recommendation is based on the IOM opinion that vitamin D basically has no bodily function beyond that of calcium homeostasis (349).

Table 8.1: RDA for vitamin D intake in various countries

Country	AI/RDA	Source
Australia and New Zealand	Infants 0-12 months: 5 ug/day (200 IU) Children 1-18 years: 5ug/day (200 IU) Adults 19-50 years: 5ug/day (400 IU) Adults 51-70 years: 10ug/day (400 IU) Adults > 70 years: 15 ug/day (600IU)	http://www.nrv.gov.au/nutrients/vitamin%20d.htm
Canada	Infants 0-12 months: 10 ug/day (400 IU) Children 1-18 years: 15ug/day (600 IU) Adults 19-70 years: 15ug/day (600 IU) Adults >70 years: 20ug/day (800IU)	http://www.hc-sc.gc.ca/fn-an/nutrition/vitamin/vita-d-eng.php
United Kingdom	Children 6 months – 5 years: 8.5 ug/day (340 IU) Adults: 10ug/day (400 IU) Adults >70 years: 10 ug/day (400 IU)	http://www.nhs.uk/Conditions/vitamins-minerals/Pages/Vitamin-D.aspx
United States of America	Infants 0-12 months: 10 ug/day (400 IU) Children 1-18 years: 15ug/day (600 IU) Adults 19-70 years: 15ug/day (600 IU) Adults >70 years: 20ug/day (800IU)	http://ods.od.nih.gov/factsheets/VitaminD-HealthProfessional/

Concerns regarding vitamin D toxicity are often cited as a reason not to increase the RDA. Whilst a negative feedback system exists for vitamin D formation by the skin in response to sunlight, no such system regulates vitamin D uptake secondary to dietary intake. A recent review by the European Food Safety Authority (EFSA) (350) concluded that daily doses of vitamin D of up to 250ug (10,000IU) IU) did not lead to persisting hypercalcaemia or hypercalciuria in adults. They also found no evidence

linking vitamin D intake and long-term health outcomes, and a wide variation in studies examining a potential link between serum 25(OH)vD concentrations and such outcomes (350).

The first attempt to define a reference range for serum 25(OH)vD was in 1971 (351). This followed fairly standard techniques: gathering a diverse population, measuring serum vitamin D levels, plotting the data and defining a reference range against a Gaussian distribution (351). In generating the data for this study, "normal" individuals were compared to a group of lifeguards, who had circulating 25(OH)vD levels 2.5 times higher than the reference population (351). It has previously been assumed that the lifeguards had abnormally high serum levels of 25(OH)vD, propagating the normal ranges espoused.

However, it has been argued that the lifeguards are actually the population with normal (i.e. physiologically healthy) serum 25(OH)vD levels, and the "normal" population were all 25(OH)vD deficient (352). An evolutionary perspective would support this theory. Skin depigmentation in response to migration patterns 50,000 years ago is thought to indicate an evolutionary response to maximise the absorption of UVB from limited sunlight in Northern areas – vitamin D deficiency leading to rickets would be a clear selection pressure causing death in childbirth in woman with rickety pelvises (352). Indeed, in populations where sun exposure is maximal, circulating 25(OH)vD levels have been reported to range from 135-225nmol/l (54-90ug/l) (352, 353).

Attempts to define physiologically normal 25(OH)vD levels have been made. At 25(OH)vD levels below 75nmol/l (30ug/l), calcium absorption by the gut is suboptimal (354). In the elderly, secondary hyperparathyroidism may occur at levels lower than this (352, 354). It has been shown that serum 25(OH)vD levels of 25nmol/l (10ug/l) are associated with a lower bone mineral density and resulting increased in the risk of fracture (355).

The current reference ranges are given in table 8.2. However, as can be seen, these are likely to be developed from flawed assumptions, and the population judged to have "insufficient" serum

25(OH)vD levels should certainly not be judged to be vitamin D replete. In addition to the flawed way in which the reference ranges have been developed, determining optimal vitamin D levels in the wider range of diseases for which low serum vitamin D is a significant risk factor is a complex process, and no conclusions have yet been reached.

Table 8.2: Reference ranges for serum vitamin D

Range	Interpretation
<25nmol/l (62.5ng/ml)	Deficient
25-50nmol/l (62.5-125ng/ml)	Insufficient
50-75nmol/l (125-187.5ng/ml)	Adequate
>75nmol/l >187.5ng/ml)	Optimal

In designing a vitamin D intervention trial for people at high risk of MS, there is an urgent need to redefine the RDA for vitamin D and enable physiological levels of supplementation. There is no convincing evidence of serious adverse events resulting from relatively high dose supplementation (in the region of 5,000-10,000 IU/day) (350), and the long-term effects from chronic vitamin D insufficiency in the population are not to be underestimated. Trials of high dose vitamin D are being performed in people with MS (356), but this intervention is clearly too late to prevent disease development.

Vaccination against infection by Epstein-Barr virus is another potential strategy. An EBV vaccine has been developed, and there has been a placebo controlled phase 2 clinical trial in 181 EBV seronegative volunteers (357). The vaccine is directed against the viral capsid glycoprotein 350 (gp350), which mediates viral entry into B-cells. The vaccine had considerable success in terms of both seroconversion against gp350 (98.7% developed anti-gp350 antibodies (95% CI 85.5%-97.9%) and preventing the development of infectious mononucleosis (mean efficacy rate 78.0% [95% CI 1.0%-96.0%]). However, it did not demonstrate any efficacy against asymptomatic infection, as measured by the development of anti-VCA antibodies (357).

The lack of protection against asymptomatic EBV seroconversion does not necessarily mean that the vaccine has no use in preventing MS. Infectious mononucleosis appears to influence MS risk independently of anti-EBNA-1 titres, and so this vaccine may have a role in modifying at least one EBV-related risk factor for MS development. Additionally, the effect of vaccination on anti-EBNA-1 titres has not been studied.

Smoking cessation strategies are now widespread, and the subject of major public health campaigns. However, 20.6% UK women and 22.8% UK males still smoke (358). There is a wealth of literature evaluating the success (or lack of) smoking prevention and cessation strategies (359-364). Smoking prevention is clearly a priority for public health in the 21st Century, and hopefully this will have an impact on MS development. In terms of MS prevention, trials in this arena would not be ethical nor feasible – interventions that are proven to reduce smoking rates should clearly be aimed at the population as a whole, not merely a proportion of those thought to be at high MS risk.

Studies to prevent MS would have to be large-scale and long-term. The modifiable environmental factors discussed above have influences on a number of diseases other than MS. It is important to ensure that longitudinal studies are set up with sufficient care taken to ensure that the large number of potential confounding factors do not render them ultimately uninformative. There remains much work to be done in confirming and validating the endophenotype concept in MS, but this study provides important initial evidence of its existence and potential utility.

Chapter 9: Future plans

There are many potential directions that future research could take. One important validatory step would be a study of people with radiologically isolated syndrome, RIS. Approximately 1/3 patients with a RIS develop clinical attacks in keeping with demyelination at longitudinal follow-up. This proportion appears to be relatively constant across a number of studies (207, 208, 365, 366). The only consistent prognostic feature for conversion to clinically apparent demyelination appears to be gadolinium enhancement on the baseline MRI (367); however only a limited number of analyses were performed across all four studies, and the average follow-up was only 2 years in two of the studies (207, 366). By calculating the MS risk score for patients with RIS and longitudinally following up the cohort for a number of years to assess conversion to clinically isolated syndrome or clinically definite MS, the use of the proposed MS risk score in a longitudinal, clinically relevant manner can be assessed. This would need to be a multi-centre, possibly multi-national approach with a relatively long follow-up period. It is not known what proportion of patients convert from RIS to CIS during a follow-up period of greater than 2 years. It may be that with a follow up of a sufficiently long duration all patients eventually convert to CIS. If this is indeed the case, this would be important to know. Such a study could allow the determination of factors that appear to influence the time to clinical conversion, enabling patients to make lifestyle changes, such as stopping smoking, that may influence their risk of future disease.

An additional future study would involve calculating the baseline MS risk score of a cohort of unaffected siblings of people with MS, followed by longitudinal follow-up over a number of years. This would enable an assessment of conversion rates, which could then be related back to the baseline MS risk score. However, the follow-up time required in such a study would be high, and thousands of siblings would be required to adequately power the study. Such a study would be considerably affected by confounding factors such as drop-out rates and behavioural changes, such as vitamin D supplementation. It would require considerable cost and administration, as it would

likely have to be a multi-national effort in order to achieve the numbers required. It would therefore seem to be sensible to combine such a longitudinal study with a placebo-controlled intervention, such as high dose vitamin D supplementation, in order to assess whether this had any effect on conversion rates.

Is an MS prevention study a realistic goal? There are two strands to answering this question. The first is whether the MS risk score detailed in this thesis would be sufficient to identify a high MS risk population such that the study could be adequately powered. Whilst the MS risk calculator appears to differentiate relatively well between people with MS and healthy controls, prospective studies are required to assess the predictive power of the risk calculator. Assuming that the MS risk calculator provides a useful predictive tool, then it could be used to inform a prevention study. In terms of interventions within a preventative study, options are limited. We cannot change the multifactorial genetic risk underpinning an individuals' MS risk at birth. As discussed above, a vitamin D supplementation study is likely to be of value, not just for MS but for a variety of diseases associated with low serum vitamin D. Vaccination against the Epstein-Barr virus is likely to have some effect on MS risk. However, as discussed above, whilst this protects against symptomatic infectious mononucleosis, it does not prevent asymptomatic seroconversion. The expected effect of vaccination on MS risk is therefore unclear, as it may only affect the risk associated with IM, or potentially the risk associated with any EBV infection. Care would need to be taken to ensure such a study was well powered to demonstrate a potentially relatively small effect on overall risk.

The secret to MS prevention may well be pre-natal intervention. This adds a further level of complexity to already challenging prevention studies. At the present time, it is unlikely that such a study is feasible, but given the pace of change in disease management and epidemiology over the past 50 years, it may well become a possibility in the future. Interventions during pregnancy, or even prior to conception, could influence the MS risk of offspring, or even of the second generation. This kind of study would involve follow-up over generations, which brings with it difficulties with dropout

rates and long-term funding. Whilst such practical challenges should not act as the only reason not to perform such a study, they are important considerations to bear in mind during study design.

With increasing knowledge surrounding epigenetics and the transmission of risk through generations, awareness is growing of the importance of prenatal factors in modifying disease risk. Coming full circle, and once again taking inspiration from the psychiatric literature, the challenges inherent in studying such diseases become apparent. Novel methods are required to study the effects of prenatal influences on complex disease development (368), which require development and validation.

The journey towards an endophenotype in multiple sclerosis is therefore only just beginning. However, as this thesis demonstrates, the concept is a valid one, and there is much potential utility to be gained in harnessing it.

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Appendices

Appendix 1: Abbreviations used in thesis

25-OHvD	25-hydroxyvitamin D
95% CI	95% confidence interval
AIT	Autoimmune thyroid disease
ANA	Anti-nuclear antibodies
AUC	Area under a ROC curve
BD	Becton, Dickinson and Company
CDMS	Clinically definite MS
CIS	Clinically isolated syndrome
CNS	Central nervous system
CRP	C-reactive protein
CSF	Cerebrospinal fluid
df	Degrees of freedom
DM	Diabetes mellitus
EAE	Experimental allergic encephalomyelitis
EBNA-1	Epstein-Barr virus nuclear antigen 1
EBV	Epstein-Barr virus
EDSS	Expanded disability status score
ELISA	Enzyme linked immuno-sorbent assay
FCS	Fetal calf serum
FLC	Immunoglobulin free light chains
FoxP3	Forkhead Box P3 (transcription factor)
GWAS	Genome-wide association studies
HC	Healthy controls
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPLC	High pressure liquid chromatography
IBD	Inflammatory bowel disease
ICH-GCP	International Conference on Harmonisation - Good Clinical Practice
IDDM	Insulin dependent diabetes mellitus
IEF	Isoelectric focussing
IgG	Immunoglobulin G
IIH	Idiopathic intracranial hypertension
IL-17	Interleukin-17
IM	Infectious mononucleosis
IU	International units
MHC	Major histocompatibility complex
MMP-9	Matrix metalloproteinase-9
MND	Motor neurone disease
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
NFh	Heavy chain neurofilament
NFl	Light chain neurofilament
NMO	Neuromyelitis optica; Devic's disease
NO	Nitric oxide

NPV	Negative predictive value
O:E	Observed:expected ratio
OCBs	Oligoclonal bands
ON	Optic neuritis
OR	Odds ratio
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PPMS	Primary progressive multiple sclerosis
PPV	Positive predictive value
RA	Rheumatoid arthritis
REC	Research ethics committee
RIS	Radiologically isolated syndrome
ROC curve	Receiver operating characteristic curve
RR	Relative risk
RRMS	Relapsing remitting multiple sclerosis
SD	Standard deviation
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
SPMS	Secondary progressive multiple sclerosis
T1DM	Type 1 diabetes mellitus
TIMP-1	Tissue inhibitor of metalloproteinase-1
UC	Ulcerative colitis
UCHL1	Ubiquitin C-terminal hydrolase
VDRE	Vitamin D response element

Appendix 2: Articles selected for inclusion in the meta-analysis of the prevalence of oligoclonal bands in MS

1. Papers used in the calculation of OCB prevalence in MS (1-29)
    a. Asian studies (excluded in conservative analysis) (30-32)
    b. Studies used in conservative analysis (1, 3, 5-10, 13, 14, 16-25, 29)
2. Papers used in the calculation of OCB prevalence in CIS (4, 9, 17, 25, 28, 33-48)
3. Papers used in the calculation of the relationship between OCB status and clinical outcomes in MS
    a. Using IEF (1, 12, 15, 18)
    b. Using alternative techniques or techniques not specified (49-54) (51-54 give outcomes in terms of EDSS)
4. Papers giving narrative results regarding the relationship between OCB status and clinical outcomes in MS (2, 3, 5, 7, 13, 18, 19, 55-59)
5. Papers used in the calculation of the relationship between OCB status and clinical outcomes in CIS
    a. Using IEF (4, 33-41, 44, 45, 47, 48)
    b. Using other techniques or techniques not specified (60-71)
    c. Studies examining outcomes in optic neuritis (all techniques for OCB detection) (47, 48, 65-71)
6. Studies used to examine the relationship between latitude and OCB prevalence in MS (1-3, 5-10, 12-25, 28-32)
7. Studies used to examine the relationship between latitude and OCB prevalence in CIS (9, 17, 25, 28, 34-48)

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Appendix 3: Participant information sheets for sibling study

a. For people with MS
b. For siblings of people with MS
c. For those siblings selected for MRI studies

Barts and The London NHS
NHS Trust

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Surgery & Anaesthesia
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Barts and the London NHS Trust
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The Royal London Hospital
Whitechapel, London
E1 1BB
Tel ext: 3303
Main switchboard: 020 7377 7000
Fax: 020 7377 7033
www.bartsandthelondon.nhs.uk

Subject Initials:

Subject Identification Number:

Patient information sheet (for people with MS)

Towards an endophenotype in multiple sclerosis

We would like to invite you to help us with a research project studying multiple sclerosis (MS), looking at both people with multiple sclerosis and their siblings (brothers and sisters). Please read the following information carefully, which explains why the research is being done and what your participation would involve. Please ask us about anything which is unclear or if you would like further information.

What is the purpose of the study?
We are studying proteins, immune signal molecules (antibodies) and white blood cells in the blood and urine of patients with multiple sclerosis and their siblings. We are looking for a common virus called the Epstein-Barr virus (the virus that causes glandular fever) in the saliva. We also want to examine the genetic code in people with MS and their siblings, to examine further whether this influences who gets the disease. We hope that through this research we can begin to understand why the relatives of people with MS have a higher risk of MS than other members of the population, and improve our understanding if the disease. We also hope that this research will help us to design new treatments and/or ideas about preventing MS.

Why have I been chosen?
We have approached you because you are someone who has been diagnosed with a multiple sclerosis and have a brother or sister who does not have MS and may be able to take part in this research.
We would like to take a blood sample from you, as well as a urine sample, a sample of cheek cells and a saliva sample. We would also like to have permission to inspect your medical notes.

Do I have to take part?
Your participation would be entirely voluntary. If you do decide to take part, we would ask you to sign a consent form. A copy of the signed consent form and patient information sheet would be given to you to keep. If you do not wish to take part, you do not have to give any reason, and this would not in any way affect the treatment or care that either you or any family members who agree to take part in this study receive in hospital or in the outpatient clinic.

What will happen to me if I take part?
If you decide to take part, we would ask you to contact your sibling(s) in order to see if they would be happy to take part in this study. If they are happy to take part, then we would be able to see you either together or at separate times, at your convenience.

We would take approximately 3 tablespoons (45ml) of your blood from a vein in your arm, which we would try to do at the same time as any other blood tests you might be having. We would also ask you to provide a urine sample, a cheek swab and a saliva sample on the same day as having the blood test. One of the blood tests being taken is to allow researchers to carry out certain genetic tests. These genetic tests do not currently allow us to predict disease. The results of these genetic tests will remain anonymous, will not be linked to my healthcare records in any way, and will only be used for research purposes.

What are the possible disadvantages and risks of taking part?
The only risks of taking part are the normal risks associated with having blood taken, such as slight bruising to the arm. There are no risks associated with providing the urine or saliva samples.

What are the possible benefits of taking part?
Your participation may help in advancing scientific knowledge about the potential causes of multiple sclerosis, although there may be no direct benefit to yourself.

Will my taking part in this study be kept confidential?
Restricted access to your medical records would be required by the research team to help interpret the results. All information which is collected about you during the course of the research would be kept strictly confidential. Any information about you which leaves the hospital would have your name and address removed so that you can not be recognised from it. Your personal data will be stored under a code rather than under your name. Only named researchers will have access to the coding sheets, which will be kept in a locked cabinet. Your GP will be informed of your participation in this study.

What will happen to the results of the research study?
We do not anticipate that the research will reveal information of immediate clinical relevance regarding any individual participant's medical care, and so we do not plan to give feedback to individuals regarding their own results. However, updates on the general progress of the research project may be given at the department's regular 'patient information days', and findings may be published in scientific academic journals within the next few years. Individual participants will not be able to be identified from any publications in scientific journals.

Who is organising and funding the research?
This research is being carried out by research doctors and scientists under the direction of Professor Gavin Giovannoni. Sections of this research are being carried out as part of PhD by Dr Dobson. The research and research workers are paid for from a variety of sources including the Medical Research Council, the Multiple Sclerosis Society of Great Britain and Northern Ireland, the NHS and funds from Queen Mary University London.

Who has reviewed the study?
Approval for this project has been granted by the East London Research Ethics Committee 2.

Storage and further use of your blood or cerebrospinal fluid samples
It is possible that not all of the blood, urine or saliva samples which you donate will be fully used up in this project. If this occurs, in order to avoid wastage of valuable samples, we might like to use them for other research projects to be undertaken in the future. Surplus samples will be transferred to a licensed human tissue storage bank, where they will be stored. Stored samples will be anonymised, but details such as age, gender, and the date on which the sample was taken will be stored. We will always maintain the confidentiality of your personal and medical information.

Future projects might include further studies on the role of antibodies or other molecules in neurological diseases. These projects may be carried out by members of Professor Giovannoni's research team, or by members of other research teams or other research institutions. All such projects will have to have been approved by research ethics committees, and the use of samples from the tissue bank is at the discretion of the custodian of that tissue bank.

Contact for further information
Further information can be obtained by contacting our research team, including our research manager Maria Espasandin, in the Neurology Clinical Trials Unit (020 7377 7000 and ext 3303).

Thank you very much for your help.

Barts and The London NHS
NHS Trust

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Surgery & Anaesthesia
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Fax: 020 7377 7033
www.bartsandthelondon.nhs.uk

Subject Initials:

Subject Identification Number:

Patient information sheet (for siblings of people with MS)

Towards an endophenotype in multiple sclerosis

We would like to invite you to help us with a research project studying multiple sclerosis (MS), looking at both people with multiple sclerosis and their siblings (brothers and sisters). Please read the following information carefully, which explains why the research is being done and what your participation would involve. Please ask us about anything which is unclear or if you would like further information.

What is the purpose of the study?
We are studying proteins, immune signal molecules (antibodies) and white blood cells in the blood and urine of patients with multiple sclerosis and their siblings. We are looking for a common virus called the Epstein-Barr virus (the virus that causes glandular fever) in the saliva. We also want to examine the genetic code in people with MS and their siblings, to examine further whether this influences who gets the disease. We hope that through this research we can begin to understand why the relatives of people with MS have a higher risk of MS than other members of the population, and improve our understanding if the disease. We also hope that this research will help us to design new treatments and/or ideas about preventing MS.

Why have I been chosen?
We have approached you because you have a brother or sister who has been diagnosed with a multiple sclerosis, and they have indicated that you may be willing to take part in this research. In order to carry out this research we need to have siblings from the same family, one of whom has MS.
We would like to take a blood sample from you, as well as urine and saliva samples and a sample of cheek cells. We will need to meet you in order to discuss your medical history, and carry out a brief examination of the nervous system.

Do I have to take part?
Your participation would be entirely voluntary. If you do decide to take part, we would ask you to sign a consent form. A copy of the signed consent form and information sheet would be given to you to keep. If you do not wish to take part, you do not have to give any reason, and this would not in any way affect the treatment or care that either you or any family members who agree to take part in this study receive in hospital or in the outpatient clinic.

What will happen to me if I take part?
If you decide to take part, then we would be able to see you either together with your sibling(s) or at separate times, at your convenience. We would take approximately 3 tablespoons (45ml) of your blood from a vein in your arm, which we would try to do at the same time as any other blood tests you might be having. We would also ask you to provide both a urine sample and a saliva sample on the same day as having the blood test. One of the blood tests being taken is to allow researchers to carry out certain genetic tests. These genetic tests do not currently allow us to predict disease. The results of these genetic tests will remain anonymous, will not be linked to my healthcare records in any way, and will only be used for research purposes.

We would also ask for your permission to be contacted at a future time in order to have an MRI brain scan. If

you were to agree to the scan, we would provide you with further information regarding it at that time. You would be free not to have the scan if you do not wish to have this done. We will only be scanning one-fifth of the participants in this study, so we will not necessarily be contacting you. If you do not wish to be contacted in the future, but still wish to take part in this research, please let one of the researchers know.

What are the possible disadvantages and risks of taking part?
The only risks of taking part are the normal risks associated with having blood taken, such as slight bruising to the arm. There are no risks associated with providing the urine or saliva samples. Those participants selected to have MRI brain scans will have the procedure explained to them in greater detail nearer the time of the scan.

What are the possible benefits of taking part?
Your participation may help in advancing scientific knowledge about the potential causes of multiple sclerosis, although there may be no direct benefit to yourself.

Will my taking part in this study be kept confidential?
All information which is collected about you during the course of the research would be kept strictly confidential. Any information about you would have your name and address removed so that you can not be recognised from it. Your personal data will be stored under a code rather than under your name. Only named researchers will have access to the coding sheets, which will be kept in a locked cabinet. Your GP will be informed of your participation in this study.

What will happen to the results of the research study?
We do not anticipate that the research will reveal information of immediate clinical relevance regarding any individual participant's medical care, and so we do not plan to give feedback to individuals regarding their own results. However, updates on the general progress of the research project may be given at the department's regular 'patient information days', and findings may be published in scientific academic journals within the next few years. Individual participants will not be able to be identified from any publications in scientific journals.

Who is organising and funding the research?
This research is being carried out by research doctors and scientists under the direction of Professor Gavin Giovannoni. Sections of this research are being carried out as part of PhD by Dr Dobson. The research and research workers are paid for from a variety of sources including the Multiple Sclerosis Society of Great Britain and Northern Ireland, the NHS and funds from Queen Mary University London.

Who has reviewed the study?
Approval for this project has been granted by the East London Research Ethics Committee 2.

Storage and further use of your blood or cerebrospinal fluid samples
It is possible that not all of the blood, urine or saliva samples which you donate will be fully used up in this project. If this occurs, in order to avoid wastage of valuable samples, we might like to use them for other research projects to be undertaken in the future. Surplus samples will be transferred to a licensed human tissue storage bank, where they will be stored. Stored samples will be anonymised, but details such as age, gender, and the date on which the sample was taken will be stored. We will always maintain the confidentiality of your personal and medical information. Future projects might include further studies on the role of antibodies or other molecules in neurological diseases. These projects may be carried out by members of Professor Giovannoni's research team, or by members of other research teams or other research institutions. All such projects will have to have been approved by research ethics committees, and the use of samples from the tissue bank is at the discretion of the custodian of that tissue bank.

Contact for further information
Further information can be obtained by contacting our research team, including our research manager Maria Espasandin, in the Neurology Clinical Trials Unit (020 7377 7000 and ext 3303).
Thank you very much for your help.

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Subject Initials:

Subject Identification Number:

Patient information sheet (MRI)

Towards an endophenotype in multiple sclerosis

We would like to invite you to help us with a research project studying multiple sclerosis (MS), looking at both people with multiple sclerosis and their siblings (brothers and sisters). Please read the following information carefully, which explains why the research is being done and what your participation would involve. Please ask us about anything which is unclear or if you would like further information. You have already provided blood, urine and saliva studies, and we would like to perform an MRI brain scan on you as well.

What is the purpose of the study?
We hope that through this research we can begin to understand why the relatives of people with MS have a higher risk of MS than other members of the population, and improve our understanding if the disease. We also hope that this research will help us to design new treatments and/or ideas about preventing MS. Through looking at the MRI scans of the brain in people with MS and their siblings, we are hoping that we can detect very subtle changes that may help us to work out how the disease begins.

Why have I been chosen?
We have approached you because you have already taken part in the first part of this study.

Do I have to take part?
Your participation would be entirely voluntary. If you do decide to take part, we would ask you to sign a consent form. A copy of the signed consent form and information sheet would be given to you to keep. If you do not wish to take part, you do not have to give any reason, and this would not in any way affect the treatment or care that either you or any family members who agree to take part in this study receive in hospital or in the outpatient clinic.

What will happen to me if I take part?
The MRI brain scans will be done at the NMR Research Unit at the Institute of Neurology, Queen Square. MRI scans are widely used in medicine and are a safe procedure that does not involve any radiation. During the examination you will lie comfortably on a moveable table that slides into a cylinder and you will hear some knocking noise that can be reduced with earplugs. You will be able to communicate with the technicians performing the study at all times and the examination can be interrupted at any time should you feel uncomfortable.

What are the possible disadvantages and risks of taking part?
MRI is very safe and used daily in clinical routine. MRI cannot be used if you have large bits of metal inside you (such as after brain surgery, or certain joint replacements), a cardiac pacemaker or if you are claustrophobic.
Although unlikely, there is a small possibility that the MRI scan may show up abnormalities within the brain that have not caused any symptoms yet. An example of this could be a small brain tumour, or changes within the blood vessels that make them more likely to bleed. Should we discover any abnormalities which have the potential to go on to cause problems in the future we would contact you, and offer you an appointment in the neurology outpatient clinic to discuss the findings and potential treatment. If the MRI brain scan does not show

any such abnormalities then we will not routinely contact you.

What are the possible benefits of taking part?
Your participation may help in advancing scientific knowledge about the potential causes of multiple sclerosis, although there may be no direct benefit to yourself.

Will my taking part in this study be kept confidential?
All information which is collected about you during the course of the research would be kept strictly confidential. Any information about you would have your name and address removed so that you can not be recognised from it. Your personal data will be stored under a code rather than under your name. Only named researchers will have access to the coding sheets, which will be kept in a locked cabinet. Your GP will be informed of your participation in this study.

What will happen to the results of the research study?
We do not anticipate that the research will reveal information of immediate clinical relevance regarding any individual participant's medical care, and so we do not plan to give feedback to individuals regarding their own results. However, updates on the general progress of the research project may be given at the department's regular 'patient information days', and findings may be published in scientific academic journals within the next few years. Individual participants will not be able to be identified from any publications in scientific journals.

Who is organising and funding the research?
This research is being carried out by research doctors and scientists under the direction of Professor Gavin Giovannoni. Sections of this research are being carried out as part of PhD by Dr Dobson. The research and research workers are paid for from a variety of sources including the Multiple Sclerosis Society of Great Britain and Northern Ireland, the NHS and funds from Queen Mary University London.

Who has reviewed the study?
Approval for this project has been granted by the East London Research Ethics Committee 2.

Contact for further information
Further information can be obtained by contacting our research team, including our research manager Maria Espasandin, in the Neurology Clinical Trials Unit (020 7377 7000 and ext 3303).

Thank you very much for your help.

Appendix 4: Data collection tool for sibling study

ID

Sib ID

Age

Gender

Month of birth

Place of birth

Lived abroad

EDSS

Date of MS dx

Type of MS

Rx

Last relapse

Last steroids

Past medical history *(thyroid/diabetes/RA/SLE/psoriasis/GI tract/sz/ME/anaemia/anything else)*

Drug history

Smoker

Ever smoked

IM

vD supplementation

Anything else

Appendix 5: The SOP for DNA extraction for 9ml whole blood, which was adjusted for the 1.5ml whole blood available

Objective
Extraction of DNA from 9ml of whole blood

Recording and Reporting of Results
This form should be printed, completed steps ticked off and any deviations from authorized protocol recorded and countersigned. A copy should be kept in the relevant process file

Method reference
BRIGHT protocol- based on salting out procedure published by miller et al 1988

Related Procedures
GC003501 Booking in of blood samples for DNA extraction
GC003502 Printing of barcode labels
GC003503 Preparation of Blood extraction solutions
GC003504 DNA Extraction from 9 ml whole blood
AF003501 DNA Extraction Batch Sheet

Operator Name;

Date;

Project ID;

Pipette IDs;

Specimen Requirements
9ml EDTA whole blood

Reagents

Name	*Supplier*	*Part Number*	*Location*
Sodium Hypochlorite solution GPR (Approx.12%w/vavailablechlorine)	VWR	301696	Flammables cupboard
96%Ethanol	VWR	104766	Flammables cupboard
18MOhmdistilleddeionisedwater	Millipore	N/A	MilliQ water purifier
500mgofProteinaseKfromTritrachiumAlbum	Sigma	P6556	Freezer1 inpost PCR room
100x	Sigma	T-9285	Chemical storage shelf
Solution1			
Solution 2			
Solution 3			

See the SOP called “preparation of blood extraction solutions” for other reagents used in this protocol

Equipment
50ml screw cap tubes
Bottles for solutions
Centrifuge
Icebox
Vortex
Water bath
Pasteur pipettes
Pipette and pipette tips
Labels

Room
Day 1 of this protocol should be carried out in the class 2 cabinet situated in the RNA room. Day 2 and 3 should be carried out in the pre-pcr room

Protocol

Make up the following solutions before starting:

Proteinase K (20 mg/ml)

Total mass of Proteinase K in bottle: 500 mg

1. To make up a final concentration of 20 mg/ml you have to add 25 ml of 18MΩ.cm MilliQ water
2. Since the bottle of proteinase K is small, it is not possible to add 25 ml straight away. So, first add 5 ml to the brown bottle of proteinase K.
3. Mix by swirling the bottle and leave to stand for a little while before transferring it to a 50 ml falcon tube.
4. Make up the volume to 25 ml.
5. Aliquot 1 ml aliquots into labeled 1.7 ml tubes and freeze at –20°C.

70% ethanol

1. Take a 1L glass bottle and a 1L measuring cylinder
2. Measure 729 ml of 96 % ethanol in the measuring cylinder
3. Make the volume up to 1L with 18MΩ.cm MilliQ water
4. Transfer volume into 1L glass bottle
5. Store the bottle in freezer 1 in the pre pcr room.

1 x TE (pH 7.5)

1. Take a 50 ml falcon tube
2. Add 4 ml of 100 x TE (pH 7.5)
3. Add 36 ml of 18MΩ.cm MilliQ water. That makes a 10 x TE (pH 7.5) dilution!
4. Take another 50 ml falcon tube
5. Add 4 ml of 10 x TE (pH 7.5)
6. Add 36 ml of 18MΩ.cm MilliQ water. That makes a 1 x TE (pH 7.5) dilution!

Protocol

This protocol is a 3-day protocol.

Day 1

Preparation

- Fill out a DNA extraction batch sheet for every batch you are extracting (AF003501 DNA Extraction Batch Sheet).
- Print out 5 replicate labels for sample tubes containing sample number and box position of where DNA will be stored after extraction
- Cool the tube centrifuge to 4°C
- Fill a large polystyrene box with ice
- Fill a 1L glass bottle with 18MΩ.cm MilliQ water
- Fill two dispojars with approximately 100 ml of sodium hypochlorite each
- Defrost proteinase K (1 tube for 16 extractions and 2 tubes for 32 extractions)

Steps

1. Defrost blood tubes in fridge o/n or for 10 min at 37°C in the water bath (The blood had been transferred into 50 ml falcon tubes upon receipt. Blood sample volumes above 9-10 ml were split in two equal volumes by pouring half of the volume into another 50ml falcon tube with identical labeling).
2. Mix the samples to resuspend the thick viscous blood.
3. Add milliQ water to make up the volume to 40ml. Vortex briefly.
4. Incubate samples on ice for 2min.
5. Centrifuge at 3000 RPM for 20min at 4°C.
6. Remove the supernatant by pouring gently to leave around 5ml. The supernatant is poured off into a dispojar containing approx. 100ml of sodium hypochlorite.
7. Add solution 1 to make up a final volume of 40ml.
8. Vortex to disperse the pellet. Do not proceed to the next step unless all the large pellets are dispersed. (This stage results in cell lysis).
9. Incubate samples on ice for 2min.
10. Centrifuge at 3000 RPM at 4°C for 15min.
11. Remove supernatant by pouring gently and save pellet.
12. Repeat steps 8 -11 using only 30ml of solution 1.
13. Gently vortex the pellet.
14. Resuspend the pellet in 11ml of solution 2.
15. Add 55μl of proteinase K (20mg/ml) to the supernatant to yield a final concentration of 100μg/ml.
16. Incubate tubes in water bath at 37°C overnight.

Important: Clean out centrifuge buckets, tube holders, class 2 cabinet and general surfaces used with Virkon which is located on the bench closest to the dishwasher on the first shelf

Day 2

Preparation

- Label the lid and the side of 50 ml falcon tubes
- Cool the tube centrifuge to 4°C

Steps

17. Take tubes out of water bath.
18. Add 4ml of solution 3.
19. Mix by inverting several times.
20. Centrifuge at 3500 RPM for 20min at 4°C (The SDS in solution 2 forms a complex with the protein, which precipitates out once NaCl is added).
21. Transfer supernatant using a 3ml Pasteur pipette to a sterile 50ml conical tube, which is correctly labeled. There are usually around 15 to 25ml of supernatant.
22. If the transferred supernatant is cloudy repeat centrifugation at 3500 RPM for 20min at 4°C. Transfer supernatant using a 3ml Pasteur pipette into a new sterile labeled 50ml conical tube.
23. Precipitate DNA by adding 96% ethanol to the supernatant up to a volume of 50ml.
24. Invert the tube at least 5 times to precipitate the DNA.
25. Store the tube at –20°C overnight.

Important: Empty sodium hypochlorite-filled blood dispojars left in hood overnight by flushing contents down the sink and flushing with excess water.

Day 3

Preparation

- Cool the tube centrifuge to 4°C

Steps

26. Take 50 ml tubes out of the freezer.
27. Spin tubes at 3100 RPM at 4°C for 15min to pellet DNA.
28. Decant off supernatant.
29. Add 15 ml ice-cold 70% EtOH per 50ml tube, invert 3 times,
30. Spin sample tubes containing pptDNA/70% EtOH at 3100 RPM at 4°C for 10min.
31. Decant off supernatant.
32. Invert the tube on tissue paper and air dry for approx. 3hrs. Mark the DNA pellet location on the outside of tube if the pellet isn't located at the base of the tube.
33. Re-suspend DNA pellet in 500µl 1xTE pH 7.5 (Resuspend the DNA in a smaller volume if you can't see a pellet or if the pellet is very small, record the volume if you add less than 500µl). Leave the tube at RT during those 48 hours.

Important: Clean out centrifuge buckets and tube holders with virkon and wipe down general surfaces used.

After DNA has dissolved

Important: NEVER vortex genomic DNA as the DNA will shear!!!

34. Quick spin 50 ml tubes to collect sample at the bottom of the tubes
35. Transfer DNA from 50 ml tubes into labeled 1.5 ml cryo-nunc tubes.
36. Rotate the nunc tubes on the rocker in the cold-room for 48 hrs before nanodropping the DNA.

Expected Results
Pure DNA for downstream analysis

Recording and Reporting of Results
The nanodrop data should be saved to a USB, and then saved in the appropriate project file in the DNA extraction from blood folder located in shares.

Sources of error/ limitations
N/A

Responsibilities
The member of staff performing the protocol is responsible

Internal Quality Assessment
N/A

Internal Quality Control
N/A

Calibration
N/A

Risk assessment
N/A

Risk Assessment

Hazard Identification#	*People/ Property Equipment at Risk*	*Current control measures*	*Risk Assessment**	*Training Requirements*
Chemical associated,				
Ethanol- flammable	Operator	Standard laboratory practice- Eye Protection, Gloves & lab coat to be worn	C3	General good laboratory Practice
Tris EDTA buffer (100x)-Irritant			C3	
Proteinase k- Harmful when inhaled and irritant			C3	
Biohazard				
DNA – potential infectious material and mutagen	Operator	Standard laboratory practice- Gloves & lab coat to be worn	C3	General good laboratory practice
Blood- potential infectious material		Work carried out in class 2 cabinets	B2	Class 2 cabinet training
Task Associated None				
Environment Associated None				

See material safety data sheets in office filing cabinet 2C for further hazard information
*A = Death or Major Injury, B= Injury resulting in >3 days Sick, C = minor Injury or minor damage
1 = very likely to occur, 2 = likely to occur, 3 = could occur

Table of minor amendments

Number	***Date***	***Page No***	***Amendment***	***Authorized by***

Appendix 6: Studies used in the calculation of the relative risk of MS associated with female sex

1. Studies used in the calculation of the relative risk of MS associated with female sex (1-69)

2. Studies used in the calculation of the relative risk of CIS associated with female sex (65, 70-101)

3. Studies used in the calculation of the relative risk of conversion to MS from CIS associated with female sex (71-74, 80, 82, 87, 91, 93)

4. Studies used in the calculation of the relative risk of RIS associated with female sex (102-107)

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Appendix 7: Demographic details of participants whose samples were used in the biomarker analysis

Please see table 4.11 for details of entire cohort

1. Samples used to examine the relationship between MS risk score and urinary FLC

   Please see table 4.11; all samples used in this analysis

2. Samples used to examine the relationship between MS risk score and urinary neopterin

	MS	Unaffected siblings	Healthy controls
Number	20	121	20
Age (mean; SD; range)	45.55 (9.21; 30-63)	47.24 (12.55; 18-75)	42.00 (13.04; 23-64)
Gender (M:F; %F)	2:18 (90% F)	38:83 (68.6% F)	8:12 (60% F)

3. Samples used to examine the relationship between MS risk score and MMP9:TIMP1

	MS	High risk score siblings	Low risk score siblings	Healthy controls
Number	20	20	20	20
Age (mean; SD; range)	45.55 (9.21; 30-63)	46.35 (10.62; 22-67)	52.42 (11.24; 31-75)	42.00 (13.04; 23-64)
Gender (M:F; %F)	2:18 (90% F)	3:17 (85% F)	8:12 (60% F)	8:12 (60% F)

4. Samples used in flow cytometry studies

	MS	High risk score siblings	Low risk score siblings	Healthy controls
Number	8	8	8	8
Age (mean; SD; range)	47.13 (8.82; 30-60)	41.63 (11.64; 22-54)	46.13 (11.03; 31-64)	43.75 (10.82; 31-61)
Gender (M:F; %F)	1:7 (87.5% F)	2:6 (75% F)	4: 4 (50% F)	4:4 (50% F)